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Down-regulation of DNA polymerase β accompanies somatic hypermutation in human BL2 cell lines

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Abstract

Somatic hypermutation (SHM) is a fundamental process in immunoglobulin gene maturation that results in increased affinity of antibodies toward antigens. In one hypothesis explaining SHM in human B cells, the process is initiated by enzymatic deamination of cytosine to uracil in the immunoglobulin gene V-region and this in turn triggers mutation-prone forms of uracil-DNA base excision repair (BER). Yet, an uncertainty with this model is that BER of uracil-DNA in mammalian cells is generally error-free, wherein DNA polymerase β (pol β) conducts gap-filling synthesis by insertion of bases according to Watson–Crick rules. To evaluate this inconsistency, we examined pol β expression in various SHM proficient human BL2 cell line subclones. We report that expression of pol β in SHM proficient cell lines was strongly down-regulated. In contrast, in other BL2 subclones, we found that SHM was deficient and that pol β expression was much higher than in the SHM proficient subclones. We also found that overexpression of recombinant human pol β in a SHM proficient subclone abrogated its capacity for SHM. These results suggest that down-regulation of the normal BER gap-filling DNA polymerase, pol β , accompanies induced SHM in BL2 cells. This is consistent with the hypothesis that normal error-free BER must be silenced to make way for an error-prone BER process that may be required during somatic hypermutation.

Keywords

B lymphocytes; Mutagenesis; Somatic hypermutation; Base excision repair; DNA polymerase β ; Activation-induced cytidine; deaminase

1. Introduction

In humoral immunity after stimulation of mature B cells with antigen presenting cells, base substitution mutations accumulate in the genes encoding the variable regions of light and heavy chain immunoglobulin molecules [1,2]. It is thought that this process is initiated with conversion of cytosine to uracil by the enzymatic activity of the activation-induced cytidine deaminase (AID) [3–5]. It has been proposed that uracil-DNA formed by AID is removed by uracil-DNA glycosylase (UDG) leaving the toxic apurinic/apyrimidinic (AP) site. In one hypothesis regarding subsequent steps, the BER pathway responds to the AP site leading to incision of the DNA strand by AP endonuclease, and in the canonical BER pathway, gap-filling and 5'-sugar phosphate removal are conducted by pol β [6]. This gap-filling step, however, is inappropriate for SHM, since pol β gap-filling synthesis is relatively error-free [7]. Clearly, if BER is involved in SHM, a DNA polymerase other than pol β must be recruited to the gapped BER intermediates involved. Studies of mouse models have shown that pol β is not required for SHM [8], while other polymerases appeared to be responsible [9,10]. Thus, while the SHM

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roles of AID, UDG, and possibly strand incision, appear clear [3,4,11], the mechanism of a mutation prone gap-filling step is uncertain since a means of removing the anti-mutagenic pol β is unknown and the interplay among multiple potential error-prone gap-filling enzymes is not clear. The presence of normal levels of pol β has been shown to be anti-mutagenic in mouse fibroblast cells; for example, the frequency of methyl methanesulfonate (MMS)-induced mutations in cells lacking pol β was higher than in isogenic wild-type cells [12]. Evidently, removal of pol β in these systems allows access during BER of an error-prone DNA synthesis processes. Interestingly, the mutagenesis in the absence of pol β was found to require the Rev1 protein [13], which presumably acts as an accessory factor for error-prone DNA polymerase (s).

In the present study, we tested the hypothesis that pol β must be silenced to make way for the induced SHM mutagenesis process. To initially explore this idea, we examined pol β expression levels in several human BL2 cell subclones that had been found to be either SHM proficient or deficient. We also created a model BL2 cell line where recombinant human pol β was stably expressed under the CMV promoter. The ability of these BL2 cell lines to conduct induced V-region mutagenesis (*i.e.*, indicative of SHM) was found to be inversely related to the pol β expression level. The implications of these results are discussed.

2. Materials and methods

2.1. Cell lines

The human Burkitt's lymphoma BL2 cell line has been described previously [14–16]. Cells were grown in RPMI medium supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA). The original BL2 cell line was sub-cloned by single cell cloning, in order to obtain 40 derivative cell lines, as described in the text. Cells were electroporated with a Flag-tagged human pol β expression plasmid [17] by a Gene Pulser electroporator (BioRad, Hercules, CA). Transfected cells were selected in the presence of 0.9 mg/ml of G418 (Invitrogen), and expression of pol β was confirmed by immunoblotting [18].

2.2. Cell extract preparation

Cell extracts were prepared as previously described [19]. Briefly, cells were washed twice with PBS at 25 °C, collected by centrifugation, and resuspended in Buffer I (10 mM Tris-HCl, pH 7.8, 200 mM KCl, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). An equal volume of Buffer II (10 mM Tris-HCl, pH 7.8, 200 mM KCl, 2 mM EDTA, 40% glycerol, 0.2% Nonidet P-40, 2 mM dithiothreitol (DTT), and protease inhibitor cocktail) was added. The suspension was rotated for 1 h at 4 °C, and the resulting extract was clarified by centrifugation at 14,000 rpm at 4 °C for use in *in vitro* BER assays. The protein concentration of the extract was determined by Bio-Rad protein assay using bovine serum albumin as a standard.

2.3. In vitro BER assay

BER assays were performed with a 35-bp oligonucleotide duplex substrate containing uracil residue at position 15. The repair reaction mixture (20 μ l) contained 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 20 mM KCl, 0.5 mM EDTA, 2 mM DTT, 4 mM ATP, 5 mM phosphocreatine, 100 μ g/ml phosphocreatine kinase, 0.5 mM NAD, 2.3 μ M [α -³²P]dCTP (specific activity, 1 $\times$ 10⁶ dpm/pmol), and DNA (250 nM). The reaction was initiated by the addition of cell extract (20 μ g), and incubation was at 37 °C. Aliquots (4 μ l) were withdrawn at the indicated times. The reaction was terminated by the addition of equal volume of DNA gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). After incubation at 75 °C for 2 min, the reaction products were separated by electrophoresis on a 15% polyacrylamide gel containing 8 M urea in 89 mM Tris-HCl, 89 mM boric acid, and 2

mM EDTA, pH 8.8. The gels were scanned using a PhosphorImager (GE Healthcare, Piscataway, NJ), and the data were analyzed by ImageQuant software. For the neutralization experiments, total cell extract (20 µg) from B22, B124 or mouse embryonic fibroblast (MEF) cells was mixed with preimmune serum or immune anti-pol β serum, and the mixtures were incubated for 30 min on ice and for an additional 5 min at 25 °C. After the incubation, the treated extracts were tested for BER as above.

2.4. In vitro UDG assay

The UDG assay was performed using a ³²P-labeled 34-bp duplex DNA that contained a G:U mismatch at position 16. The 34-bp substrate was prepared by annealing two oligodeoxynucleotides: 5'-CTGCAGCTGATGCGCUGTACGGATCCCCGGGTAC-3' and 5'-GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG-3'. The uracil-containing strand was labeled with [γ -³²P]ATP using T4 polynucleotide kinase and then annealed to the G-containing strand by heating at 90 °C for 3 min and slowly cooling to 25 °C. Unincorporated [γ -³²P]ATP was removed by using a MicroSpin™ G-25 column (GE HealthCare) according to the manufacturer's suggested protocol. The reaction mixture in a final volume of 10 µl contained 50 mM HEPES, pH 7.5, 20 mM KCl, 0.5 mM EDTA, 2 mM DTT, 50 nM ³²P-labeled DNA, 20 µg cell extract, and with or without 2 units of UDG inhibitor termed UGI (New England BioLabs, Ipswich, MA). The reaction mixtures were incubated for 10 min at 37 °C. The AP site generated by enzymatic uracil removal from the substrate was hydrolyzed by adjusting the mixture to 0.1N NaOH and incubating for 5 min at 75 °C. The reaction product was separated from substrate by electrophoresis in a 15% denaturing polyacrylamide gel. A PhosphorImager (GE HealthCare) was used for scanning the gels.

2.5. Immunoblotting

Immunoblotting analysis was performed as described previously [13]. Cell extracts, as indicated in figure legends, were separated by Nu-PAGE 4–12% Bis-Tris mini gel (Invitrogen), and the proteins were transferred electrophoretically to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.5% (v/v) Tween-20 (TBST) for 1 h, washed once with TBST, and incubated for 2 h with appropriately diluted primary antibody against rat pol β (18S) [18], human pol ι [20], human pol λ [21], and anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Alpha Diagnostic Intl. Inc., San Antonio, TX). After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The activity of immobilized horseradish peroxidase-conjugate was detected by enhanced chemiluminescence (ECL), as suggested by the manufacturer (Pierce, Rockford, IL).

2.6. Cytotoxicity studies

Cytotoxicity was determined by growth inhibition assays similar to that described previously [13]. Suspensions of B cells, stably transfected with the pol β construct (clone B124) or control cells (clone B22) were treated with a range of concentrations of MMS in growth medium without G418. MMS was added directly to the medium at the time of the experiment. After 1 h exposure, cells were washed twice with Hanks' balanced salt solution (HyClone, Logan, UT), and resuspended in fresh medium without G418 to a density of 10,000 cells/ml (2 ml per well) in 6 well dishes, then incubated in a 5% CO₂ incubator for 4–5 days. Cells (triplicate wells for each agent concentration) were counted and results were expressed as the number of cells in agent-treated wells relative to untreated wells (*i.e.*, %control growth).

2.7. Real time PCR

Fresh CD19⁺ B cells were purchased from Stem Cells Technologies, Inc., Palo Alto, CA. Total RNA from these and other cells was isolated with Trizol (Invitrogen), according to the

manufacturer's recommendations. Then, total RNA (1–5 µg) isolated from cells was treated with RNase-free DNase I (Ambion, Austin, TX) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen) in the presence of 100 ng oligo(dT)18 primer, according to the manufacturer's suggested protocol. Real time PCR with SYBER green detection was performed using an ABI Prism 7700 thermocycler with fluorescence detections (Applied Biosystems, Inc., Foster City, CA). Real time PCR primer sets for human and mouse pol β amplification were purchased from SuperArray, Inc., Frederick, MD. For amplification of an internal standard, a real time PCR primer set for β-actin was purchased from Ambion, Inc. Appropriate “no reverse transcriptase” and “non-template” controls were included in each 96-well reaction plate. Relative levels of pol β mRNA were calculated based on the level of RNA in the internal control (β-actin).

2.8. Immunohistochemical analysis

For immunohistochemical analysis, the slides of normal adult tonsil and testis tissue were purchased from US Biomax, Inc., Rockville, MD. Tissue sections were deparaffinized with xylene and hydrated through an alcohol rinse series to 1× automation buffer (Biomedica, Inc., Foster City, CA). After heat inducible epitope retrieval in a Decloaker™ (Biocare Medical, Inc., Concord, CA), slides were treated with 3% H₂O₂ for endogenous peroxidase blocking. Then, the tissue sections were reacted with affinity purified polyclonal anti-8 kDa pol β antibody raised against the N-terminal 8 kDa domain of rat pol β [22], at a dilution 1:1000 for 1 h. Antibody–antigen detection was performed with the ABC kit (Vector Laboratories, Inc., Burlingame, CA). The end product was visualized by exposure to 3,3-diaminobenzidine (Dako, Corp., Carpinteria, CA).

2.9. Mutagenesis in B cells

The mutational process was activated *in vivo* by the combined crosslinking of surface immunoglobulin with CD19/CD21 receptors [23]. Cells (2×10^6) were incubated with 4 µl of biotinylated anti-human IgM (Pharmingen, San Diego, CA), 20 µl of anti-human CD19 (Southern Biotech., Birmingham, AL), 40 µl of phycoerythrin–anti-CD21 (Pharmingen) in 500 µl of RPMI medium for 20 min at 4 °C. Then, cells were washed twice with fresh RPMI medium and incubated with 15 µl of streptavidin-conjugated magnetic beads (Invitrogen) in 500 µl of RPMI medium at 4 °C for 20 min with rotation. Activated cells were incubated at 37 °C overnight in complete RPMI medium. Genomic DNA from treated cells was isolated with DNAzol (Invitrogen). The variable region (V-region) of Ig genes was amplified from genomic DNA with Pfu DNA polymerase (Stratagene, La Jolla, CA) using the primers, V_H4-n 5'-GAGGCTGCCTCTGATCCCAG-3' and J_H5-n 5'-CCTGGCAAGCTGAGTCTCCC-3'. The conditions for PCR amplification were 94 °C for 1 min, 35 cycles at 94 °C for 45 s, 60 °C for 90 s, and 72 °C for 2 min. The PCR products were cloned with the Zero Blunt TOPO cloning kit (Invitrogen). Plasmid DNA was eventually extracted from individual bacterial colonies and sequenced in an automated sequencer (ABI Prism 310 genetic analyzer, Applied Biosystems).

2.10. AID RT-PCR

Total RNA from induced B22, B24, and B124 cells was isolated with TRIzol (Invitrogen). RNA (2 µg) isolated from cells was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) in the presence of 100 ng oligo(dT)18 primer, according to the manufacturer's suggested protocol. The AID mRNA was amplified from three-fold dilutions of cDNA with GoldTaq I DNA polymerase (Perkin-Elmer, Wellesley, MA) using the primers, AIDf 5'-AGGCAAGAAGACACTCTGG-3' and AIDr 5'-GTGACATTCCTGGAAGTTGC-3'. The conditions for PCR amplification were 94 °C for 1 min, 21 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min. The control G3PDH mRNA was amplified under the same conditions

with primers, G3PDHf 5'-TCCACCACCCTGTTGCTGTAG-3' and G3PDHr 5'-GACCACAGTCCATGCCATCACT-3'.

3. Results

3.1. Pol β protein level by immunoblotting

In the experiments to be described, we made use of the Burkitt's lymphoma cell line, BL2, for the following reasons. First, the cell line has surface markers of centroblast stage B cells [14, 15], indicating that these cells are immune competent. Second, the V-region of the IgG heavy chain gene undergoes only a low rate of spontaneous mutagenesis, whereas mutagenesis can be induced ~10-fold by addition of cell surface receptor ligands, and this is considered reflective of SHM [14–16]. In preliminary studies, we found variability among samples of BL2 cells with regard to the amount of V-region mutations observed after induction. To take advantage of this fluctuation, we isolated 40 individual single-cell BL2 subclones and then characterized them. We chose to first analyze 18 of the BL2 subclones for pol β expression level using immunoblotting (Fig. 1A). Different levels of pol β were found among these BL2 subclones, but they could be grouped into four categories (Table 1), including those with negligible pol β expression (two clones), and those with a detectable level of pol β using 40 μ g of cell extract (three clones). Even in the three clones with a relatively high level of pol β (B5, B7, and B24), the amount was much lower than in our laboratory reference line of MEF cells (Fig. 1A and Table 1). The cell extract from clone B22, though not clearly positive for pol β expression in the survey experiment shown in Fig. 1A, was re-examined using larger amounts of extract (Fig. 1B). The results revealed that clone B22, indeed, contained pol β , but at a lower level (5- to 10-fold) than clone B24, and a much lower level than in the reference MEF line.

3.2. V-region mutations in BL2 clones B7, B10, B22, B24, and B25

Next, we examined the question of a possible correlation between the capacity for induced V-region mutagenesis and pol β expression in clones B7, B10, B22, B24, and B25. Analysis of V-region mutations in uninduced clone B22 cells revealed only two G to C transversions, equivalent to approximately 2×10^{-4} mutations/bp (data not shown). Induction of clones B22 and B10 cells increased the frequency of V-region mutations to 14.5×10^{-4} mutations/bp (Table 2). In this case, mutations were predominantly base substitutions in G:C base pairs (74 of 77 total); there were four 1-bp deletions and two 2-bp deletions. This was in agreement with features of the SHM process in B cells, where 62% of mutations were G:C to A:T transitions [16]. Similar analysis of clone B7, B24, and B25, on the other hand, failed to reveal any difference between induced and uninduced mutations in the V-region: the frequency of mutations in induced and uninduced studied clones was similar to that seen in uninduced clone B22, *i.e.*, 1.9×10^{-4} mutations/bp (Table 2).

3.3. In vitro BER capacity

Since a form of error-prone BER has been hypothesized to be involved in SHM, we next characterized the *in vitro* BER capacity of cell extract from clone B22. Although the pol β level was deficient in clone B22, we wanted to know if these cells were capable of supporting a robust level of extract-based BER. This was uncertain, as it is well known that MEF deficient in pol β have only a low capacity of *in vitro* BER in the assay used here [17]. We compared the *in vitro* BER capacity of clone B22 extract with that of reference extracts from wild-type MEF. For this analysis, a 35-bp oligonucleotide duplex containing a uracil residue opposite guanine was incubated with the cell extracts; incorporation of [32 P]dCMP into the DNA substrate, in place of uracil, was measured under initial rate conditions (Fig. 1D). In spite of much lower expression of pol β in clone B22, similar levels of BER activity were found with extracts from clone B22 and wild-type mouse fibroblast (Fig. 1D). These results suggested that

clone B22 had a considerable capacity for BER, even in the absence of high expression of pol β . Evidently, other BER DNA polymerases, such as pol λ and pol ι , are expressed at a high enough level to support BER in the absence of strong pol β expression.

3.4. Overexpression of pol β in mutation proficient clone B22

While clones B22 and B24 differed in pol β expression and also differed markedly in induced V-region mutational phenotypes, this association was only correlative. Therefore, we could not exclude the possibility that proteins other than pol β may have been responsible for the V-region induced mutational difference. To further explore a link between pol β expression and the absence of induced V-region mutations, we developed a derivative of clone B22 stably expressing a transgene for human pol β driven by the CMV promoter. Expression from the pol β transgene in various transformants was ascertained by immunoblotting. A clone with expression of recombinant pol β was selected for further study and designated as clone B124. Since the pol β open reading frame was fused to the Flag epitope in these experiments, the recombinant enzyme had slightly decreased mobility compared with native pol β (~5 kDa) in immunoblotting analysis (Fig. 2A).

Since pol β expression was down-regulated in clone B22, we characterized the expression level of two other DNA polymerases implicated as back-up enzymes in mammalian BER [21,24]. In Fig. 2B, it is shown that expression of both pol ι and pol λ was strong in clones B22 and B124. In contrast to the results for pol β expression, the level for each of these enzymes was similar across the three cell lines, and the levels also were similar to that seen in the MEF extract (not shown).

Next, to confirm that the expressed recombinant pol β was functionally active, we first compared the *in vitro* BER capacity of extracts isolated from the parental clone B22 and the transformant, clone B124. The BER activity of clone B124 extract was higher than that of clone B22 (Fig. 2C), consistent with expression of active pol β in these cells. To address the question whether this BER capacity in the clone B124 extract was driven by pol β *versus* another polymerase, we made use of a neutralizing antibody against human pol β (Fig. 2D) [25]. This anti-pol β antibody decreased repair to a near-background level with the clone B124 extract, suggesting that most of the repair synthesis was due to pol β in this case. Activity in the extract from MEFs, used as a reference, also was due mainly to pol β , as expected [21]. In contrast, the BER activity in the extract of clone B22, where the level of pol β was low, was only partially reduced (~30%) by the antibody, suggesting that repair synthesis in this extract was mainly due to other polymerases. Finally, it is well established that a pol β phenotype *in vivo* is protection of cells against the cytotoxic effects of alkylating agents, such as MMS [17,26]. Thus, we compared clones B22 and B124 for their resistance to MMS-induced cytotoxicity. Exponentially growing cells were treated with various doses of MMS, were washed and grown in fresh medium for several days, and the surviving cells were counted. Clone B124 was found to be more resistant to MMS than the parental clone B22 (Fig. 2E). Taken together, these results indicated that the expressed recombinant pol β was functionally active.

3.5. V-region mutations in BL2 clone B124

We next examined V-region mutations in induced and uninduced clone B124. Analysis of 138 independently derived V-region sequences revealed only 10 base substitutions corresponding to approximately $\sim 1.7 \times 10^{-4}$ mutations/bp (Table 2) in the induced cells, and a similar level was observed in the uninduced cells. Thus, the capacity for induced V-region mutagenesis had been abrogated by ectopic expression of pol β in these cells. These results are consistent with our conclusion noted above and indicate that expression of pol β in BL2 clone B24 cells diminished the capacity for induced V-region mutagenesis (Fig. 3 and Table 2).

The SHM process is known to be initiated by AID and requires UDG activity [4,11]. Differential expression of these enzymes could have had an impact on the frequency or spectrum of mutations accumulated within the V-region after induction. We examined expression of these enzymes in clones B22, B24, and B124. AID protein expression in extracts of these cells was undetectable in our hands by immunoblotting. However, the AID mRNA could be measured by semi-quantitative RT-PCR. Amplification of the AID cDNA from mRNA isolated from activated B22, B24, and B124 clones demonstrated a similar level of expression (Fig. 4A). Next, we examine UDG activity in cell extracts prepared from these clones. The extracts appeared to have a similar level of UDG activity (Fig. 4B). The activity detected in each case was due to UDG, since activity was completely blocked by UGI. Therefore, the failure to observe V-region mutations in clones B24 and B124 was not due to lower expression of the enzymes required in the initial steps of the BER process, compared with clone B22.

3.6. Expression of pol β in tonsils

Pol β expression in tissue sections of human tonsil was analyzed by immunohistochemistry using an antibody that is specific to pol β [22]. Sections of human testis were used as a positive control; deep nuclear staining was observed in the early spermatid and staining was also noted in primary spermatocytes, and spermatogonium (Fig. 5e), as was reported previously [27–29]. In contrast, tissue sections treated with preimmune serum showed no antibody staining (Fig. 5d and f). We did not observe pol β expression in germinal centers of tonsil tissue (Fig. 5a and c). However, there was scattered nuclear staining of cells in the connective tissue zone outside of the germinal centers (Fig. 5a and b). To test for pol β expression level in normal human B cells, we measured pol β mRNA in the CD19⁺ population of peripheral blood lymphocytes (PBL). A real time PCR assay revealed that the mRNA level of this polymerase in PBL was similar to that in B22 cells and approximately 20-fold lower than in MEF cells (Fig. 6). We conclude from these results with normal human B cell tissue (tonsil) and PBL that pol β expression was lower than in adjacent connective tissue and reference specimens used, *i.e.*, testis and MEF cells, respectively.

4. Discussion

We made use of a human cell culture system for study of the induced SHM process. The approach involved subclones of BL2 Burkitt's lymphoma cells and DNA sequence analysis to reveal immunoglobulin V-region induced mutagenesis [1]. We found an inverse relationship between the pol β expression level in BL2 subclones and the amount of V-region mutations after induction through activation of surface receptors (Fig. 3). Hence, a clone expressing a relatively high level of pol β failed to undergo induced mutagenesis in the V-region, whereas a clone with a relatively low level of pol β expression was V-region mutagenesis proficient. Another cell line derived through transfection of a pol β expression vector into the mutagenesis proficient clone was now found to be deficient in V-region induced mutagenesis. The results are consistent with the hypothesis that pol β is down-regulated in BL2 cells to make way for error-prone DNA synthesis mechanisms inherent in a BER leading to SHM.

SHM is dependent on expression of a suite of selected genes in the immune competent B cell compartment of germinal centers. It is known, for example, that deletion of genes for two of the BER enzymes in mouse models inactivates the SHM process: AID-deficient mice and UDG-deficient mice have a complete loss of, and altered SHM, respectively [11,30,31]. In human B cells, down-regulation of DNA pol β eliminated induced SHM [32,33], and in a mouse model, mutation of the pol β gene resulted in lower induced mutagenesis in the V-region [9, 34]. It is also known that expression of AID is up-regulated in germinal center cells, and this may promote targeted mutagenesis in the V-region. Since the anti-mutagenic BER polymerase

pol β has high affinity for the gapped intermediates produced during BER and is expressed strongly in many cell types, it is expected that pol β expression in germinal center B cells would block the potential for SHM through error-free gap-filling DNA synthesis in BER. Therefore, it is logical to expect either that BER is not involved or that mechanisms for down-regulation of pol β may be fundamental to a BER related process of SHM, just as mechanisms for up-regulation of AID expression are considered fundamental. Since mechanisms of blocking pol β are unknown, some authors have favored mechanisms of SHM that do not involve BER gap-filling [34].

The difference in V-region induced mutagenesis observed here among BL2 clones B22, B24, and the derivative B124 did not appear due to be a difference in expression of pol ι , pol λ , and AID, as revealed by immunoblotting of the extracts. These enzymes were evaluated because it has been shown that pol ι is involved in V-region mutagenesis in human BL2 cells [3,16,32,33], and, in addition pol λ another member of the group of BER proficient DNA polymerases [35] can serve as a backup enzyme for pol β in BER [21]. Both of these polymerases carry intrinsic dRP lyase activities that may be important in short patch BER [24,36–38]. We analyzed expression of these enzymes by immunoblotting and found no significant differences in the BL2 clones studied here. While we could not detect AID protein expression by immunoblotting, we tested for expression of AID mRNA using a quantitative RT-PCR assay. The results demonstrated that there were no major differences in AID expression in clones B22 and B24 (Fig. 4).

Even though we found a reduction in pol β expression in the clone B22 line, the extract from these cells was proficient in *in vitro* BER. Obviously, DNA polymerases other than pol β appeared to be responsible for the gap-filling involved in this BER activity, and this was consistent with the results of the anti-pol β neutralization study shown in Fig. 2. The identity of the polymerase(s) responsible for this synthesis was not evaluated here.

Accumulation of mutations is a balance of two inconsistent processes: formation of the lesion, and the removal of this lesion by a DNA repair pathway. Uracil is constantly generated in the genomic DNA either by dUTP insertion by replicative DNA polymerases or by spontaneous and enzymatic deamination of cytosine. Spontaneous deamination of the cytosine alone results in generation of roughly 60–120 uracil residues per human genome per day [39]. However, the frequency of mutations in human cells is low because the majority of these lesions are efficiently repaired by BER [40]. In human B cells, uracil residues generated in the region corresponding to the variable portion of the heavy and light immunoglobulin genes are either repaired in an error-prone way or are fixed in following rounds of replication, diversifying the antibody repertoire. Thus, the G:C to A:T mutagenesis observed here could arise by error-prone uracil-DNA BER by insertion of T opposite G, most likely by pol ι [41,42], or by replicative incorporation of A opposite U. We favor the former hypothesis because it explains the need to down-regulate pol β to make way for an error-prone polymerase and is consistent with the observed requirement for pol ι .

In conclusion, we examined the expression of pol β , an enzyme that plays a key role in BER. Our results showed a reduced expression level of pol β in the SHM proficient B cell lines studied, and the CD19⁺ population of human B cells. Ectopic expression of this polymerase in BL2 cell lines that were proficient for SHM, severely disturbed this process. Based on our results and results from other groups, we suggest that expression of AID is not sufficient for SHM through replicative synthesis past uracil-DNA. Instead, the process appears to require additional events mediated through error-prone BER. The mechanism and control of these BER events will be the topic of further investigation.

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Fig. 1.

Analysis of BL2 cell line clones (*i.e.*, subclones). (A) Screening of BL2 clones for expression of pol β by immunoblotting. Photographs of blots after enhanced chemiluminescence are shown. Cell extract protein (40 μ g) from each individual clone was separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with a mouse monoclonal antibody (18S) against pol β or anti-G3PDH antibody, as a loading control. Lane 1, 10 μ g of MEF cell extract; lanes 2–10 and 12–20, 40 μ g of cell extracts from individual BL2 subclones; lane 11, 3 ng purified human pol β . (B) Titration of the B22 subclone extract for detection of pol β expression. Various amounts of B22 extract were separated by SDS-PAGE and analyzed as in (A). Lanes 1–4, respectively, 24, 12, 6, and 3 ng of purified human pol β and lanes 5–8 contain 20, 40, 80, and 120 μ g of extract, respectively. (C) *In vitro* BER time

courses showing the BER capacity of B22 and MEF cell extracts. The BER reaction mixtures were incubated for 5 min (lanes 1 and 4), 10 min (lanes 2 and 5), and 20 min (lanes 3 and 6). A photograph of a PhosphorImager scan of the ligated BER products (35-mer) is shown. A schematic line-diagram of the 35-bp oligonucleotides duplex is also shown. C* illustrates the incorporation of [^{32}P]dCMP into DNA after uracil-DNA repair. Experiments were conducted as described in Section 2.

Fig. 2.

Characterization of the B22 subclone and the B124 transformant clone. (A) Immunoblot analysis showing expression of Flag-tagged human pol β in BL2 cells. Cell extract (40 μg) protein from B124 (lane 2), B22 (lane 3) or purified pol β (4 ng) (lane 1) was separated by SDS-PAGE and transferred to nitrocellulose membrane; the membrane was probed with anti-pol β antibody, as in Fig. 1. The migration of recombinant pol β in the B124 cell extract was slightly slower than that of native pol β because the enzyme contained a Flag tag. (B) Immunoblotting analysis of extracts from subclones B22, B24, and B124 for expression of pol ι and pol λ. Extract protein (20 μg) was separated by SDS-PAGE, transferred to nitrocellulose and analyzed with antibodies to human pol ι, human pol λ, or G3PDH as a loading control. (C) *In vitro* BER time courses showing the BER capacity of B22 and B124 cell extracts. The BER reaction mixtures were incubated for 2 min (lanes 1 and 4), 5 min (lanes 2 and 5), 10 min (lanes 3 and 6), or 20 min (lanes 4 and 8) at 37 °C. A photograph of a PhosphorImager scan of the gel region corresponding to the ligated BER products (35-mer) is shown. (D) Neutralization of pol β-mediated BER activity in cell extracts by anti-pol β antibody. The conditions and product analyses were as described in Section 2. Cell extract (20 μg) from B22, MEF, or B124 cells was incubated with pre-immune IgG or immune anti-pol β IgG, as indicated; the mixtures were incubated for 30 min on ice and for an additional 5 min at 25 °C. BER products, PhosphorImager (PI) arbitrary units, formed in the presence of pre-immune IgG were set to 100% and were 2139 PI units/min/μg protein, 3046 PI units/min/μg protein, and 11,735 PI units/min/μg protein, respectively, for B22, MEF, and B124 extracts. The BER activities in the anti-pol β containing reaction mixtures were plotted as a percentage of the activities in the reaction mixtures with pre-immune IgG. BER activity was taken in each case from the linear relationship observed between incubation time and product formation. (E) Cytotoxicity assays of B22 and B124 cells after treatment with various concentrations of MMS (for 1 h). Survival

curves represent B22 (open circle) and B124 (closed circle) cells, as shown. Values presented are the means $\pm$ S.E.M. of five independent experiments. Within one experiment, means were calculated from triplicate values. Experiments were conducted as described in Section 2.

Fig. 3.

Summary of results illustrating the inverse relationship between mutation frequency and pol β expression level. Clone B124 (overexpressing recombinant human pol β) is represented by +++++; category IV clones B7, B24, and B25 are represented by ++; and category I clones, B10 and B22, are represented by ±.

Fig. 4.

Analysis of AID mRNA expression and UDG activity in extracts from clones B22, B24, and B124. (A) AID mRNA was quantified by RT-PCR as described in Section 2. Amplified cDNA was prepared from activated clones, as indicated, and separated by agarose gel electrophoresis. A photograph of an agarose gel is shown. (B) UDG activity of B22, B24, and B124 cell extracts was measured as described in Section 2. Uracil-containing oligonucleotide substrate was incubated with cell extract without (lanes 2, 4, and 6) or with UGI (lanes 3, 5, and 7). The reaction mixtures were incubated for 10 min at 37 °C, and products were separated by 15% denaturing PAGE. A photograph of a PhosphorImager scan of a gel is shown.

Fig. 5. Immunohistochemical detection of pol β in human tonsil. Immunohistochemical staining of normal adult tonsil (a–d) and, as a positive control, of normal adult testis (e and f). Formalin-fixed and paraffin-embedded tissue sections were stained either with preimmune IgG (d and f) or affinity purified antibody (IgG) against the 8 kDa domain of pol β (a–c, and e). Two regions (b and c) of human tonsil (a) were enlarged: one was from the germinal center (GC) region (c) and the other was outside of the germinal center region (b), as illustrated by the square boxes. Dark brown staining (a and b) shown by two solid arrows indicated nuclear staining of pol β . Such dark brown staining was not observed in germinal center cells (c), but was observed in the surrounding stromal cells (b).

Fig. 6.

Analysis of expression of pol β mRNA in normal human B cells by real time PCR. Real time PCR analyses of pol β mRNA level in CD19⁺ B cells, MEF cells, and clone B22 were performed. Values of pol β mRNA expression were calculated relative to an internal standard (β -actin mRNA). The values are mean $\pm$ S.E.M. of three independent experiments. Experiments were conducted as described in Section 2.

Table 1Pol β expression in BL2 clones (*i.e.*, subclones)

Category	Relative amount (pg/ μ g) ^a	Clone
I	1–2	B10 and B22
II	2–5	B11, B12, B13, B14, B18, B21, and B23
III	5–10	B6, B25, B26, B32, and B37
IV	10–20	B5, B7, and B24

^aRelative amount of pol β was from immunoblot analysis. For reference, the amount of pol β in MEF was approximately 75 pg/ μ g of extract.

Table 2

Mutation frequency in activated BL2 clones (*i.e.*, subclones)

B10 and B22 (pol β deficient)				B7, B24, and B25 (pol β proficient)				B124 (plus pol β)			
Mutation type	N	%	Mutation frequency, $\times 10^{-5}$	Mutation type	N	%	Mutation frequency, $\times 10^{-5}$	Mutation type	N	%	Mutation frequency, $\times 10^{-5}$
G:C to A:T	48	62	90.4	G:C to A:T	4	44	8.3	G:C to A:T	1	10	1.7
G:C to T:A	3	4	5.6	G:C to T:A	2	22	4.1	G:C to T:A			6.8
G:C to C:G	23	30	43.3	G:C to C:G	3	33	6.2	G:C to C:G	4	40	1.7
A:T to G:C	1	1	1.9	A:T to G:C				A:T to G:C	1	10	1.7
A:T to C:G	1	1	1.9	A:T to C:G				A:T to C:G	3	30	5.1
A:T to T:A	1	1	1.9	A:T to T:A				A:T to T:A	1	10	1.7
Total	77		145.0	Total	9		18.6	Total	10		17.2

Mutation frequency in the variable region of the IgM gene in induced BL2 cells. Results with six cell lines are shown: B10 and B22 (pol β deficient); B7, B24, and B25 (pol β proficient); B124 (plus pol β). Mutation frequencies were calculated after analysis of 126 (B22, B10), 115 (B7, B24, B25), and 138 (B124) of IgM V(D)J regions, respectively. Experiments were conducted as described in Section 2.