Impact of DNA ligase IV on nonhomologous end joining pathways during class switch recombination in human cells

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Class switch recombination (CSR) is a region-specific, transcriptionally regulated, nonhomologous recombinational process that is initiated by activation-induced cytidine deaminase (AID). The initial lesions in the switch (S) regions are subsequently processed and resolved, leading to recombination of the two targeted S regions. The mechanisms by which repair and ligation of the broken DNA ends occurs is still elusive. Recently, a small number of patients lacking DNA ligase IV, a critical component of the nonhomologous end joining (NHEJ) machinery, have been identified. We show that these patients display a considerably increased donor/acceptor homology at Sμ–Sα junctions compared with healthy controls. In contrast, Sμ–Sγ junctions show an increased frequency of insertions but no increase in junctional homology. These altered patterns of junctional resolution may be related to differences in the homology between the Sμ and the downstream isotype S regions, and could reflect different modes of switch junction resolution when NHEJ is impaired. These findings link DNA ligase IV, and thus NHEJ, to CSR.
assay (8), we report that the nature of switch recombination junctions is altered, showing either a marked shift to utilization of microhomologies (≥4 bp) (μ–α), or an increased frequency of insertions (μ–γ). This implies that DNA ligase IV is directly involved in CSR, and when it is defective, alternative pathways may be required to repair DSBs.

RESULTS AND DISCUSSION

Switching to IgA and IgG in Lig4D patients

Genomic DNA was purified from peripheral blood samples from healthy blood donors and the two Lig4D patients (411BR and 2304). To determine whether switch recombination was affected by a lack of functional DNA ligase IV, individual switch junctions were amplified using a previously described PCR protocol (8–10). The numbers of Sμ–Sα fragments were determined from 10 PCR reactions run in parallel using DNA from each individual and can be used as an estimation of the number of clones that have switched to IgA. As shown in Fig. 1, when 30 ng of template DNA was added in each PCR reaction, only two weak bands were generated from each of the Lig4D patients (Fig. 1, 411BR, lanes 2 and 3, and 2304, lanes 3 and 7), whereas at least 14 distinct Sμ–Sα fragments were amplified from the control. The experiments were repeated independently several times and the intensity of the smear and the numbers of bands obtained from the patients (ranging from 0 to 2) were consistently less compared with those of the controls.

To estimate the degree of reduction in the number of switched clones in the patients, we subsequently performed the same PCRs on a series of dilutions of the DNA templates from normal individuals (n = 5) and patients. The average number of Sμ–Sα fragments generated from controls correlated with the amount of DNA template (r = 0.84, P < 0.05) and 30 ng of DNA input from the patient was equivalent to 0.37–0.74 ng (41–81-fold less) of the control samples (Fig. S1 B, available at http://www.jem.org/cgi/content/full/jem.20040772/DC1). No switch fragment could be amplified from the two patients when DNA input was <30 ng (Fig. S1 B). Taking into account that one patient had lymphopenia (an approximately threefold reduction) and that the proportion of B cells was low in the remaining lymphocytes (411BR: 2.6% of PBLs are B cells, normal controls showing 5–17%), the number of clones that have switched to IgA appears to be reduced by ∼2–14-fold in this patient. To further estimate the relative proportion of B cells in the peripheral blood, we amplified the VH-JH rearrangements from the DNA samples used in the CSR assay. 411BR had a reduced level of rearranged VH genes (∼4–8-fold less), which is comparable to the estimation from the lymphocyte count (∼20–fold less), whereas 2304 had close to the normal level of rearranged VH genes. Additional experiments performed using mixtures of cells with a known proportion of B cells from normal controls (n = 2) showed that one to two switch fragments could still be amplified when “normal” B cells constituted as few as 0.15–0.45% of PBL (5–17-fold fewer B cells than present in 411BR). Thus, the reduced number of switch fragments we observed in the patients is not only due to fewer B cells but also appears to reflect a reduced number of cells that had switched to IgA.

Patient 411BR had a reduced serum IgG2 and very low levels of anti-Pneumococcal antibody titres before and after vaccination (6 and 14 IU/ml, respectively; normal range, 20–200), which would support the notion of a reduced efficiency of switching in this patient. Indeed, we could not amplify any Sμ–Sy2 fragment from this patient using a Sy2-specific primer, whereas in controls (n = 3), an average of 16 fragments could be amplified under the same conditions. Similarly, only one Sμ–Sy2 fragment was amplified from the second patient. The numbers of Sμ–Sy1 and Sμ–Sy3 fragments were also lower in both patients than those from controls (Fig. S1 C and not depicted), suggesting a parallel reduction in the number of clones that have switched to IgG in these patients.

Sμ–Sα junctions exhibit long microhomology in Lig4D patients

The amplified Sμ–Sα fragment sequences were aligned to the germline Sμ and Sα sequences to define the switch junctions. Altogether, 50 Sμ–Sα junctions from the Lig4D patients were characterized (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20040772/DC1). Five sequences from each patient are shown in Fig. 2. Switching to α1 occurred more often than to α2 both in patients and controls (60 and 66%, respectively). No sequential switching through Sγ was observed (7% in controls), further supporting the notion that there may be a reduction in the efficiency of CSR in these patients, as sequential switching would involve more than one recombination event.

Next, we analyzed the actual usage of microhomology at the switch junctions. There was a strikingly high degree of overlap between the Sμ and Sα sequences in switch junctions derived from the Lig4D patients (9.8 ± 7.5 vs. 1.8 ± 3.2 nucleotides in controls; Student’s t test, P < 0.00001). Almost
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all junctions (29 out of 30; 97%) displayed microhomology of ≥1 bp (i.e., at least one nucleotide is shared by both the Sμ and Sα regions; Table I). Moreover, 60% of the junctions exhibited a long microhomology of ≥7 bp (Table I). This was in sharp contrast with Sμ–Sα junctions derived from normal donors (Table I), where approximately half (42%) showed no sequence homology at all and only a minority of the junctions showed a microhomology of ≥7 bp (10%). When one mismatch was allowed at either side of the switch junction, >80% of the switch junctions from the patients were flanked by ≥10/11 bp of imperfect repeats (Table I). The shift in using long microhomologies or imperfect repeats in the Sμ–Sα junctions from Lig4D patients was even more pronounced than previously observed in patients with other disorders involving DNA repair (ataxia-telangiectasia [A-T]), Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia–like disorder (ATLD; references 10, 11).

Significantly more Sμ breakpoints from the patients (73 vs. 31% in controls) were located in the part of the amplified Sμ region that shows the highest degree of homology with Sα1 or Sα2 (position 275–760; χ² test, P < 0.001; Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20040772/DC1). Similar shifts have been described previously in A-T, NBS, and ATLD patients and PMS2 knockout mice, where increased junctional microhomology is also observed (11, 12). Subsequently, we compared all the junctions with breakpoints within bp 275–760 of the Sμ region from Lig4D patients and controls (22 and 47 junctions, respectively); the junctions from Lig4D patients still exhibited a significantly higher degree of microhomology in all the categories (P < 0.01 or P < 0.001). This suggests that clustering of breakpoints in the part of the Sμ where homology to Sα is most pronounced is due to the need for donor/acceptor homology in resolving the DSBs associated with IgA switching.

Another feature of the Sμ–Sα junctions from the Lig4D patients was a reduction in the number of mutations or insertions at, or around, the breakpoints. We have demonstrated previously that mutations or insertions frequently occur close to CSR junctions (≥15 bp; references 11, 13). However, this was rarely seen in the Sμ–Sα junctions from Lig4D patients, where only 3 out of 30 fragments (10%) showed mutations or insertions in this region (compared with 56% in controls; χ² test, P < 0.001).

Point mutations in the germline Sμ region, which are probably generated by an AID-dependent process (14, 15),

Figure 2. Sequences of Sμ–Sα junctions. The Sμ and Sα1 or Sα2 sequences are aligned above and below the recombined switch junctional sequences. Microhomology was determined by identifying the longest region at the switch junction of perfect uninterrupted donor/acceptor identity (boxed with solid lines). Imperfect repeat was determined by identifying the longest overlap region at the switch junction by allowing one mismatch on either side of the breakpoint (the extra nucleotide identity beyond the perfectly matched sequence identity is boxed by dotted lines). The Sμ and Sα breakpoints for each switch fragment are indicated by ▽ and △, respectively, and their positions in the germline sequences are indicated on the top or below the arrowheads. The number of base pairs involved in microhomology and imperfect repeat (numbers indicated in parentheses) for each junction is shown at the bottom right of each switch junction.
can also be observed in the recombinated Sμ region, away from (starting 15-bp upstream) the switch junctions (16). The pattern of these mutations is similar to that in the V regions (thus referred to as SHM like). However, they are clearly different from those at, or close to, the switch junctions and occurred at a much lower frequency, suggesting the involvement of a different repair mechanism (11, 16). In Lig4D patients, SHM-like mutations were observed at a much lower frequency (1.8/1,000 bp) as compared with those from controls (6.4/1,000 bp, χ² test, P < 0.001). Furthermore, the general pattern of base substitutions was altered, occurring mainly at A-T sites (56 vs. 37% in controls) and showing a preference for transitions (78 vs. 59% in controls).

The RGYW/WRCY (R = A or G, Y = C or T, W = A or T) motifs serve as hotspots for the SHM-like mutations in the recombinated Sμ region (16, 17) in normal controls. In Lig4D patients, a similar rate of targeting was observed (72 vs. 70% in controls). Most of the mutations in these motifs are due to transitions (92 vs. 60% in controls; χ² test, P < 0.005) and the last nucleotide of the motif was more often mutated in the patients than in controls (38 vs. 5%, χ² test, P < 0.001). Although a diminished rate of CSR or proliferative response may explain the reduction of SHM-like mutations, this cannot explain the bias for targeting within the RGYW/WRCY motifs and the altered pattern of mutations. Thus, like ATM (16), DNA ligase IV is also, directly or indirectly, involved in the generation of SHM-like mutations in the Sμ region.

We further analyzed a few Sμ–Sα junctions (n = 8) generated from experiments with a low amount of template DNA or a reduced proportion of B cells from normal controls. The nature of these junctions was similar to those amplified under standard conditions (30 ng of DNA from peripheral blood cells). Therefore, the aberrant switch junctions derived from patients are not likely to be due to any bias arising from PCR amplification when specific target sequences are rare.

Sμ–Sγ switch junctions show an increased proportion of 1-bp insertions in Lig4D patients
A high proportion of Sμ–Sγ junctions (n = 59) from normal blood donors display a microhomology of ≥1 bp; a frequency similar to, or even higher, than at Sμ–Sα junctions (68 vs. 58%; Table I). However, fewer Sμ–Sγ junctions showed a microhomology of ≥4 bp (5 vs. 20%) or ≥7 bp (0 vs. 10%) as compared with the corresponding Sμ–Sα junctions (Table I).

### Table I. Characterization of Sμ–Sα and Sμ–Sγ junctions

<table>
<thead>
<tr>
<th>Perfectly matched homology</th>
<th>Imperfect repeats (with one mismatch)</th>
<th>Total no. of S junctions</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1 bp</td>
<td>≥4 bp</td>
<td>≥7 bp</td>
</tr>
<tr>
<td>Controls</td>
<td>90 (58%)</td>
<td>31 (20%)</td>
</tr>
<tr>
<td>Lig4D</td>
<td>29 (97%)</td>
<td>22 (73%)</td>
</tr>
</tbody>
</table>

| Sμ–Sγ junctions            |                                      |                         |                         |          |                          |                      |                          |          |                          |      |
|----------------------------|--------------------------------------|-------------------------|-------------------------|----------|-------------------------|                      |                          |----------|-------------------------|      |
| Controls                   | 40 (68%)                             | 3 (5%)                  | 0 (0%)                  | 0 (0%)   | 10 (17%)                | 2 (3%)                | 0 (0%)                  | 0 (0%)   | 6 (10%)                  | 59   |
| Lig4D                      | 19 (56%)                             | 4 (12%)                 | 0 (0%)                  | 0 (0%)   | 12 (35%)                | 3 (9%)                | 0 (0%)                  | 0 (0%)   | 11 (32%)                 | 34   |

*Statistical analysis was performed using χ² test, comparing the number of junctions from the Lig4D patients with the corresponding category of junctions from normal controls. For Sμ–Sα junctions, in all categories, significant differences (P < 0.001) between patients (bold numbers) and controls were observed. For Sμ–Sγ junctions, a significant difference (P < 0.05) between patients and controls was observed only in the category with 1-bp insertion (bold). Parts of the data from controls have been described previously (references 9–11). The Sμ–Sα and Sμ–Sγ junctions were derived from 17 and 33 normal controls, respectively.

Mutations or insertions are frequently associated with the Sμ–Sγ junctions (≥15 bp) in controls (47%). However, the pattern of nucleotide changes seems to be slightly different from those associated with the Sμ–Sα junctions, with more mutations occurring at G/C sites (91 vs. 83%) and, although transitions are preferred (54 vs. 45% in Sμ–Sα junctions), G→T nucleotide changes occur more often at the Sμ–Sγ junctions (27 vs. 9%; χ² test, P < 0.01). Together, these data suggest that Sμ–Sγ junctions may be resolved differently from Sμ–Sα in normal controls.

Subsequently, we cloned and sequenced 36 Sμ–Sγ fragments from the Lig4D patients. 34 of these fragments were unique in their junctional sequences and the majority of the junctions were due to switching to IgG1 (n = 18) or IgG3 (n = 13). There was a trend toward an increased usage of microhomologies (≥4 bp) or imperfect repeats (≥4/5 or ≥7/8 bp) in the Lig4D patients, but not to a statistically significant degree (Table I).

Mutations occurred at a normal frequency around the Sμ–Sγ junctions in cells from the Lig4D patients (15 out of 34 fragments; 44 vs. 41% in controls) and no statistically significant alteration in the mutation pattern was observed. However, Sμ–Sγ junctions from patients exhibited insertions (1 bp) at the breakpoints (Table I) more frequently than from controls (χ² test, P < 0.05).

### End joining pathways in CSR in Lig4D patients
A number of alternative NHEJ pathways have been proposed in yeast and vertebrates (18). In the former, a Mre11/Rad50/Rad1-dependent pathway characterized by microhomology-mediated end joining, which is only partially dependent on the yeast DNA ligase IV homologue, Dnl4, has been described previously (19). In vertebrates, substantial evidence exists for an alternative end-joining mechanism that is
independent of the “classical” NHEJ proteins. Thus, end joining during V(D)J recombination or in various plasmid assays is inefficient in Ku, DNA ligase IV, or XRCC4-deficient systems, and the recovered junctions occur principally at short direct repeats with several (usually ≥4 or ≥6) base-pairs of microhomology flanking the DSB (20–23).

The data presented here demonstrate that the resolution of CSR junctions is significantly altered when DNA ligase IV function is impaired. Although the Sμ–Sα junctions show considerable donor/acceptor microhomology, far more than that seen in healthy individuals, the Sμ–Sγ junctions show only a trend toward an increase in homology compared with controls. The predominantly used, error-prone, end joining pathway in CSR appears to be impaired in Lig4D patients, and the Sμ–Sα regions are joined by an alternative, error-free end joining mechanism, involving microhomology. This alternative mechanism may also be used in recombination of Sμ–Sγ regions in the patients, although to a much lower degree. It is possible that yet another alternative pathway is used for joining of the Sμ–Sγ regions in Lig4D patients, where 1-bp insertions are frequently introduced. It is worth noting that even in normal controls, the Sμ–Sα and Sμ–Sγ regions are resolved differently, with varying degrees of dependence on microhomology and different patterns of junctional mutations, which suggests that multiple pathways (dominant and alternative) are involved in resolving the initial lesions in the S regions. The balance in utilization between these putative different pathways might depend not only on the factors available but also on the degree of homology between the S regions. It remains unclear if the alternative mechanisms proposed are totally independent of DNA ligase IV, as some residual level of functional protein might still be present because our patients carry hypomorphic mutations.

A further characteristic of the Sμ–Sα junctions in Lig4D patients is the lack of previously observed junctional mutations. This feature has previously been observed in Sμ–Sα junctions from A-T patients (ATM defective; reference 10), but not those derived from NBS and ATLD patients (NBS1 and Mre11 defective, respectively; reference 11), or Sμ–Sγ junctions from PMS2-deficient mice (12). Therefore, the end joining mechanism, used in Sμ–Sα recombination when ligase IV or ATM is defective, is partially different from the alternative NHEJ pathways demonstrated in yeast, where mutations and insertions are observed along with an increased usage of microhomology (19). DNA ligase IV is probably not only necessary in the final ligation step in CSR, but also, as suggested previously by in vitro studies, required for alignment-based gap filling (24). This process involves noncomplementary ends, is error-prone or imprecise, and could well be accompanied by recruitment of an error-prone polymerase such as pol η, which has previously been implicated in the generation of mutations in the switch region (25).

In conclusion, the involvement of DNA ligase IV in CSR is clearly demonstrated by the altered pattern of in vivo recombination at the switch junctions. Three components of NHEJ (DNA-PKcs, Ku70, and Ku80) have previously been implicated in CSR. However, as both Ku and DNA PKcs display additional NHEJ-independent functions (3), it is uncertain whether the CSR defects observed in Ku and DNA-PKcs knockout mice are due to an impairment of the NHEJ pathway. Because DNA ligase IV and XRCC4 have no reported roles outside NHEJ, our study links DNA ligase IV and, therefore, NHEJ, to CSR.

MATERIALS AND METHODS

Patients. Two out of the four DNA ligase IV–defective (Lig4D) patients characterized previously (7) were included in this study. Patient 411BR carries three homologous alterations in LIG4 and the second patient (2004), from an independent family with two affected siblings, is a compound heterozygote for two truncating mutations (7). Patient 411BR was reported to have pancytopenia (7), at the time of sampling having 0.37 × 10⁹ cells/ml of lymphocytes with a lowered proportion (2.6%) of B cells. Pretreatment Ig level determination showed that IgG2 was below the normal range; the patient is currently being treated with regular immunoglobulin infusions. Patients 2004 had chronic respiratory infections and “bone marrow failure” although the lineages affected were not clearly stated at the time. Previous records on Ig levels or lymphocyte counts were not available from this patient. The institution review boards at the Karolinska Institute approved the study.

Amplification of switch (Sμ)–Sα and Sμ–Sγ fragments. Genomic DNA was purified from peripheral blood cells from patients and healthy blood donors. The amplification of Sμ–Sα fragments was performed as described previously (8, 10). In brief, two pairs of Sμ and Sγ-specific primers were used in a nested PCR assay (Fig. S1 A). The number of Sμ–Sα fragments was determined from 10 reactions run in parallel using DNA (30 ng per reaction) from each individual and represents random amplification of in vivo–switched clones. The PCR error rate was estimated previously (0.9/1,000 nucleotides; reference 8).

CD19-positive B cells were isolated from peripheral blood mononuclear cells from normal individuals using Dynabeads M-450 CD19 (Dynal). Genomic DNA was extracted from the positively isolated B cells and the negatively isolated non–B cell populations from the same individual. The VH-FK3-JH rearrangements were PCR amplified as described previously (26).

The Sμ–Sγ fragments were amplified as described previously (9) and two new primers, SY1 specific (5′-ACGTACACGGCCCTAGGCTGTC-TGTT-3′) and SY2 specific (5′-GTCTCAGTGTGGCTGCTCTG-CTCAGTAT-3′) were applied to allow detection of switching to IgG1 and IgG2, respectively.

Analysis of the Sμ–Sα and Sμ–Sγ junctions. The PCR-amplified Sμ–Sα and Sμ–Sγ fragments were purified, cloned, and sequenced as described previously (8). The breakpoints were determined by aligning the switch fragment sequences with the Sμ (X54713)/Sα1 (L19121)/Sα2 (AF030305) or Sμ/Sγ1 (U39937)/Sγ2 (U39934)/Sγ3 (U39935)/SY4 (Y12547-52; reference 27) sequences. Microhomology was defined as successive nucleotides that were shared by both the Sμ and Sα or Sγ regions at the switch junction (without mismatches). The term imperfect repeat was used when one mismatch was allowed adjacent to the breakpoint. Insertion was defined as a nucleotide at the breakpoints that was not identical to either of the switch regions. Mutation close to, or at, the breakpoint was defined as a nucleotide change within 15 bp on either side of the switch junction. Polymorphisms in regions. Mutation close to, or at, the breakpoint was defined as a nucleotide change within 15 bp on either side of the switch junction. Polymorphisms in

Online supplemental material. Fig. S1 describes the PCR strategy for amplification of Sμ–Sα and Sμ–Sγ fragments and estimation of the input of DNA templates. The sequences for all the Sμ–Sα junctions are presented in Fig. S2. Distribution of the Sμ–Sα breakpoints is given in Fig. S3. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20040772/DC1.
REFERENCES


