A new gene involved in DNA double-strand break repair and V(D)J recombination is located on human chromosome 10p

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V(D)J recombination, accountable for the diversity of T cell receptor- and immunoglobulin-encoding genes, is initiated by a lymphoid-specific DNA double-strand break. The general DNA repair machinery is responsible for the resolution of this break. Any defect in one of the known components of the DNA repair/V(D)J recombination machinery (Ku70, Ku80, DNA-PKcs, XRCC4 and DNA ligase IV) leads to abortion of the V(D)J rearrangement process, early block in both T and B cell maturation, and ultimately to severe combined immune deficiency (SCID) in several animal models. A human SCID condition is also characterized by an absence of mature T and B lymphocytes, and is associated with an increase in sensitivity to DNA-damaging agents (RS-SCID). None of the above-mentioned genes are defective in these patients, arguing for the likelihood of the existence of yet another unknown component of the V(D)J recombination/DNA repair apparatus. Athabascan-speaking (SCIDA) Navajo and Apache Native Americans have a very high incidence of T–B– SCID. The SCIDA locus is highly linked with markers on chromosome 10p, although the exact molecular defect has not been recognized in these patients. We show here that cells with the SCIDA defect are impaired in the DNA repair phase of V(D)J recombination similarly to RS-SCID, precisely an absence of V(D)J coding joint formation. Moreover, genotyping analysis in several RS-SCID families corroborates a linkage of the RS-SCID locus to the SCIDA region on chromosome 10p. These results demonstrate the presence of a new essential DNA repair/V(D)J recombination gene in this region, the mutation of which causes RS-SCID in humans.

INTRODUCTION

Immunoglobulin and T cell receptor genes are composed of variable (V), diversity (D) and joining (J) segments, which undergo somatic rearrangements prior to their expression. This process is completed by the V(D)J recombinase and can be roughly divided into two phases (reviewed in ref. 1). During the initial step, the lymphoid-specific proteins Rag1 and Rag2 (2,3) recognize recombination-specific sequences (RSSs) that flank all V, D and J gene segments, and introduce a DNA double-strand break at the border of the RSS (4,5). Subsequently, the general DNA repair machinery is recruited to resolve the double-strand break and complete the V(D)J rearrangement (6–10). Among the factors involved in the second step are the Ku70/Ku80/DNA-PK complex, XRCC4 and DNA ligase IV proteins. Several murine models, including murine scid (11), have highlighted the critical role that all these factors play in the development of lymphoid cells. Indeed, when not embryonic lethal, their targeted inactivation results in a severe combined immunodeficiency disease (SCID) characterized by an absence of both B and T lymphocytes (12–19). When considering the genes involved in the second phase of V(D)J recombination, the immunological phenotype is also accompanied by a more general defect in DNA double-strand break repair that translates into an increased sensitivity of non-lymphoid cells to DNA-damaging agents such as γ-rays.

In humans, ~20% of SCID patients harbor a T–B– phenotype characterized by an absence of circulating mature T and B lymphocytes but the presence of natural killer cells (20), arguing for a possible defect in the V(D)J recombination process (OMIM 202500). These patients can be further divided into two groups according to the response of their cells to ionizing radiation. Some patients are defective in the early phases of V(D)J recombination, carry mutations in either the Rag1- or Rag2-encoding genes (21,22), and do not demonstrate an increased radiosensitivity. Other patients, on the contrary, are also characterized by an increased radiosensitivity of their bone marrow cells and fibroblasts (23–25), supporting the hypothesis of a defect in one of

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the Rag1 and Rag2 genes, in contrast to the Rag1/Rag2 mutated SCID patients (23). However, none of the currently known DNA repair/V(D)J recombination factors are affected in RS-SCID patients (23).

There is an increased prevalence of T–B– SCID, referred to as SCIDA. SCID and control fibroblasts were transfected with Rag1, Rag2 and the V(D)J recombination substrates phRecCJ and phRecSJ. Extrachromosomal substrates were subjected to PCR amplification for analysis of coding joints (120 bp) and signal joints (190 bp). Digestion of signal joint PCR products results in two fragments of 100 and 90 bp. SCIDA cells are defective in coding joint formation (lane 4), but demonstrate normal signal joints (lanes 5 and 6).

The genes involved in DNA repair. We refer to these patients as RS-SCID. Fibroblasts from these patients cannot carry out coding joints during V(D)J recombination of extrachromosomal substrates on transfection with the Rag1 and Rag2 genes, in contrast to the Rag1/Rag2 mutated SCID patients (23). However, none of the currently known DNA repair/V(D)J recombination factors are affected in RS-SCID patients (23).

RESULTS AND DISCUSSION

Defect in V(D)J recombinase activity in SCIDA fibroblasts

The DNA repair phase of V(D)J recombination can be examined in fibroblasts on transfection of Rag1/Rag2 expression plasmids together with an extrachromosomal substrate. The two reporter plasmids phRecCJ and phRecSJ are composed of a LacZ gene interrupted by a stuffer DNA fragment flanked on both sides by RSSs. When unrearranged, the LacZ gene is non-functional and bacterial colonies are white on X-gal-containing media. A functional LacZ gene is reconstituted on V(D)J-mediated recombination, and leads to blue colonies when reintroduced into bacteria. The presence of blue colonies therefore attests to an efficient V(D)J recombination process. phRecCJ analyzes the formation of coding joints, whereas phRecSJ is specific for the formation of signal joints. We demonstrated previously that RS-SCID patients are profoundly impaired in V(D)J coding joint formation, whereas the formation of signal joints is not affected (23). As shown in Table 1, no blue colonies were recovered from the SCIDA and P16 cell lines when using the coding joint-specific reporter plasmid phRecCJ. On the contrary, recombined plasmids (blue colonies) were obtained on transfection of the signal joint-specific reporter phRecSJ in these cell lines. The frequency of signal joint formation was constantly lower in the SCIDA cell line compared with the RS-SCID cells. In front of this quantitative difference, we analyzed the nature of the signal joint in SCIDA cells. The formation of signal joints creates a diagnostic ApalI restriction site at the junction when it is precise. Polymerase chain reaction (PCR) analysis of coding and signal joint formation following transfection revealed an absence of coding joint (Fig. 1, lane 4) in SCIDA as expected, but the presence of a signal joint-specific band (lane 5) that could be digested further with ApalI (lane 6). This attested to the precise nature of the signal joints in SCIDA, as already demonstrated in other human RS-SCID (23). The absence of coding joint formation is a characteristic of all the RS-SCID cases that we have tested to date. These results therefore demonstrate that SCIDA and P16 belong to the RS-SCID group of patients with respect to their inability to undergo DNA repair following V(D)J recombination.

### Table 1. V(D)J recombination assay in fibroblasts from SCIDA and RS-SCID

<table>
<thead>
<tr>
<th></th>
<th>phRecCJ (coding joints)</th>
<th>phRecSJ (signal joints)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blue color</td>
<td>Total</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>55</td>
<td>4,800</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>33</td>
<td>6,000</td>
</tr>
<tr>
<td>SCIDA (AK5760)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>0</td>
<td>120,000</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0</td>
<td>140,000</td>
</tr>
<tr>
<td>RS-SCID (P16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1</td>
<td>45,000</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0</td>
<td>100,000</td>
</tr>
</tbody>
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aR(coding joints) = 3 × [(blue color)/(total)] × 1000.
bR(signal joints) = [(blue color)/(total)] × 1000.
Figure 2. Pedigrees of three families with RS-SCID. Black circles and squares denote affected individuals. A diagonal line indicates a deceased individual. The genotypes for 11 microsatellite markers on chromosome 10p are represented below each tested individual. Markers showing homozygosity in patients are shaded. The region between D10S223 and D10S1763 is the minimal interval homozygous in every affected patient.
Genetic linkage between RS-SCID and chromosome 10p markers

As a first step towards the cloning of the gene defective in RS-SCID, we undertook a genome-wide scan in family F1.A using microsatellite polymorphic markers with a mean distance of 10 cM between markers. Among several chromosomal regions that showed evidence for linkage, with inherited homozygosity in the three affected children, one appeared to correspond to that of the newly described SCIDA locus on chromosome 10, centered on D10S191. A further detailed pairwise linkage analysis was carried out in three RS-SCID consanguineous families using markers close to D10S191 (Fig. 2 and Table 2). The region of homozygosity in the affected patients extended from D10S223 to D10S1763. Moreover, the very same homozygous haplotype spanning five markers from D10S223 to D10S1763 was inherited in affected patients from the two related families F1.A and F1.B. This result further indicated this region as being linked to RS-SCID. Finally, the highest LOD scores, 8.01 and 7.71, were
observed for markers D10S1664 and D10S191, respectively, at a recombination fraction (θ) of 0.00. The LOD score for D10S191 reaches 12.2 when considering the previous score defined in SCIDA populations (29). This region comprises the previously assigned SCIDA locus location. These results demonstrate that the defective genes responsible for RS-SCID and SCIDA are most likely the same, although we cannot formally rule out the existence of two closely linked genes involved in V(D)J recombination and DNA repair. The Rag1 and Rag2 locus location certainly provides the best example of such a possibility as these two loci are co-located on human chromosome 11 and mutation in either one leads to the same SCID phenotype. Our linkage analysis could not, however, narrow down the region. Interestingly, a similar haplotype covering four markers was present in F1.A/B and F3, arguing for a possible founder effect in these two families.

Our overall conclusion is, therefore, that a new DNA repair/V(D)J recombination gene is located on human chromosome 10 between D10S191 and D10S1763, the mutation of which causes RS-SCID. Whether or not this gene accounts for all the cases of RS-SCID awaits its characterization and further sequencing in non-consanguineous families.

MATERIALS AND METHODS

Subjects

All T-B-SCID patients described in this study were characterized by a complete absence of peripheral B and T lymphocytes, whereas natural killer cells were present. Patients P5, P11, P12 (kindred F1.A in Fig. 2) and P15 (family F3) were described previously for their increased radiosensitivity and impaired V(D)J recombination (23–25) and therefore belong to the RS-SCID subgroup. P16 (family F2) was not analyzed for its radiosensitivity, but was included in the RS-SCID group based on the V(D)J recombination defect (this study), as is the SCIDA patient (AK5760). Families F1.B and F1.C are related to F1.A (Fig. 2) and the affected children exhibited the same T-B-SCID phenotype. Although the V(D)J assay was not performed on cells from these patients, we assume that they inherited the same genetic defect. F1, F2 and F3 are Turkish, Italian and French families, respectively. Primary fibroblast cell lines were obtained from skin biopsies and pseudo-immortalized with SV40 as described (23). Cell lines were cultured in RPMI 1640 (Glutamax; Gibco BRL, Gaithersburg, MD) supplemented with 15% fetal calf serum. Informed consent was obtained from patients’ parents prior to this study.

Genetic analysis

Genotyping for the polymorphic microsatellite markers D10S1216, D10S1430, D10S1705, D10S223, D10S1725, D10S1664, D10S191, D10S1763 and CHLC.GATA6E06.200 was performed using primer sequences and PCR conditions available through the Genome Database (http://www.gdb.org). Forward primers of each pair were end-labeled with \([\gamma-32P]ATP\). PCR products were analyzed on a 5% acrylamide denaturing gel. A set of individuals with known alleles was used as a sizing control. Haplotypes were constructed so as to minimize the number of intrafamilial recombination events. Pairwise analysis was performed using the MLINK software of the LINKAGE package. Allele frequencies for all markers were kept equal.

V(D)J recombination assay

The V(D)J recombination assay was carried out in SV40-transformed fibroblasts as described previously (23). Briefly, 5 × 10^6 exponentially growing cells were electroporated in 400 μl of complete culture medium with 6 μg of Rag1- and 4.8 μg of Rag2-encoding plasmids together with 2.5 μg of either phRecCJ (coding joint) or phRecSJ (signal joint) V(D)J extrachromosomal substrates. Forty-eight hours after transfection, extrachromosomal DNA was prepared by rapid alkaline lysis and used for transfection of DH10B bacteria. The percentage of recombination was determined by plating bacteria in the presence of X-gal and IPTG and then counting blue colonies. Recovered extrachromosomal DNA was subjected to PCR amplification using T3 and T7 flanking primers and run on 2% agarose gels. To analyze the nature of the signal joints, PCR products obtained following transfection with phRecSJ were digested with ApoLI.

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