A congenital mutation of the novel gene *LRRC8* causes agammaglobulinemia in humans

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A girl with congenital agammaglobulinemia and minor facial anomalies lacked B cells in peripheral blood: karyotypic analysis of white blood cells showed balanced translocation, t(9;20)(q33.2;q12). In the current study, we isolated a novel gene, *leucine-rich repeat–containing 8* (*LRRC8*), at the translocation site on chromosome 9. It has four transmembrane helixes with one isolated and eight sequentially located leucine-rich repeats (LRRs) and constitutes a new protein family. It is expressed on T cells as well as on B-lineage cells. Translocation truncates the *LRRC8* gene, resulting in deletion of the eighth, ninth, and half of the seventh LRR domains located close to the C-terminal. The truncated form of the *LRRC8* gene is transcribed with sequences from the noncoding region adjacent to the truncated seventh LRR. Protein products derived from the truncated gene are coexpressed on white blood cells with the intact LRRC8 protein from the untranslocated allele. Transplantation experiments with murine bone marrow cells that were forced to express the truncated LRRC8 show that expression of the truncated protein inhibited B cell development. These results indicate that LRRC8 is responsible for the B cell deficiency in this patient and is required for B cell development.

Introduction

B cell development is achieved with sequential and combined effects of participating molecules. B-lineage commitment from common lymphoid progenitors is initiated with a transcription factor, PAX5, which presumably suppresses differentiation toward other lineages (1–3). EBF and E2A are two other B lineage–specific transcription factors that work at an early developmental stage (4, 5). At a later developmental stage, the pre-B receptor complex and its related molecules SYK, BLNK, and BTK are indispensable for B cell development (6–11). Interactions with intrinsic factors within the cells have not yet

been fully revealed. Humoral factors derived from the hematopoietic microenvironment are also significant for B cell development during early and late developmental stages (12): stromal cell–derived factor-1 is indispensable in both humans and mice (13, 14), while IL-7 is essential in mice (15) but not in humans (16, 17). In addition, bone marrow stromal cells play significant roles via contact with B-precursor cells as well as secretion of humoral factors such as the cytokines and chemokines mentioned above. However, the majority of participating molecules residing on stromal cells still remain undetermined.

Agammaglobulinemia is a congenital syndrome characterized by a defect of B cells with preserved T cell function. This syndrome seems to be caused by abnormalities of molecules involved in B cell development and/or proliferation. The molecules responsible for the syndrome are therefore presumed to be intrinsic factors acting within or on B-precursor cells, or factors residing in the microenvironment that support B cell development. Thus, their delineation will contribute to a further understanding of the B cell ontogeny. The most common cause of agammaglobulinemia is a defect of the *BTK* gene (18, 19). Mutations of the *BTK* gene are found in approximately 80% of patients with agammaglobulinemia (20). Recently, defects of BLNK and some subunits of the

pre-B receptor complex, μ heavy chain, λ5/14.1, and CD79a, have been reported in patients with agammaglobulinemia (6–9, 11). However, such abnormalities are displayed in a minority of cases, and pathogenesis remains unresolved in the remainder. Thus, other unknown molecules essential for development and/or proliferation of B cells probably exist (21).

A girl with agammaglobulinemia and minor facial anomalies lacked B cells in peripheral blood. Karyotypic analysis of her white blood cells showed a balanced chromosomal translocation, t(9;20)(q33.2;q12). Expression of BTK in her white blood cells was unaffected, and all other previously mentioned molecules known to be responsible for agammaglobulinemia were not located on the translocation site. In the current study, we isolated a novel gene, *leucine-rich repeat–containing 8* (*LRRC8*), and its truncated form that resulted from the chromosomal translocation. Transfer of the truncated gene into murine hematopoietic stem cells inhibited B cell development. Our data indicate that LRRC8 plays a significant role in B cell development, thereby adding a novel candidate to the group of molecules essential for B cell development.

Methods

Patient. A 17-year-old girl with congenital agammaglobulinemia lacked B lymphocytes in peripheral blood and showed epicanthic folds, mild hypertelorism, high-arched palate, and lowered ears; no family member exhibited immunodeficiency. The absolute count of her peripheral lymphocytes was 3,300 per microliter, and the proportions of CD20-, CD2-, CD4-, and CD8-positive lymphocytes were 0.6%, 97.0%, 56.0%, and 34.7%, respectively. G-banded chromosomal analysis of her leukocytes showed 46, XX, t(9;20)(q33.2;q12), de novo, i.e., her parents had no chromosomal abnormality. Informed consent was obtained from the patient and her parents.

Isolation of the LRRC8 gene. For FISH analysis, we chose bacterial artificial chromosome (BAC) and P1-derived artificial chromosome (PAC) clones on the long arm of chromosome 20 based on the Contig map from the Sanger Centre and the physical map of GeneMap'99 from the National Center for Biotechnology Information, both purchased from BACPAC Resources (Oakland, California, USA). FISH analysis was performed in a commercial clinical laboratory. The ET1033 probe was a PCR product amplified with primers ET1033s and ET1033a. Inverse PCR involved genomic DNA digestion with BamHI and BglII with self-ligation using T4 DNA ligase, followed by PCR amplification with an inverse pair of primers on chromosome 20 to uncover the mutation of der20 (22). For cycle sequencing, templates were labeled with Terminator Ready Reaction Mix, and cDNA sequences were read with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Applera Corp., Foster City, Utah, USA). mRNAs were isolated from peripheral mononuclear cells obtained from the patient and healthy donors. The primers for RT-PCR were NG1044 on exon 1 and PH221 on exon 2 for wild-type, and NG459 on exon 1 and TWIN2 on chromosome 20 for mutant.

Detection of the LRRC8 protein. Rabbit polyclonal antibodies (poAbs) against amino acids 342–589 of human LRRC8 were made by a commercial laboratory and used as primary antibodies for Western blot or flow cytometry analysis. The poAb's also reacted with the murine ortholog. We carried out Western blot analysis using an HRP-conjugated donkey anti-rabbit antibody as a secondary antibody, and ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). For flow cytometry analysis (FACSCalibur; Becton Dickinson and Co., Franklin Lakes, New Jersey, USA), cells were preincubated with goat serum and then incubated with the poAb's against LRRC8. After several washes, stained cells were reacted with a phycoerythrin-conjugated goat anti-rabbit Ig antibody (Sigma-Aldrich, St. Louis, Missouri, USA). Lineage-specific mAb's (Becton Dickinson and Co.) used in this study were as follows: murine granulocytes, Gr-1; monocytes, Mac-1; T cells, CD3; and human and murine B-lineage cells, CD19 and B220, respectively.

Preparation of the retroviral vectors MIY and MutY. MIY is a mock vector, and MutY is a vector producing a truncated form of the LRRC8 protein. The truncated form of the *LRRC8* gene was synthesized by PCR amplification using an NF9 and AIN2 primer pair. Ecotropic Phoenix packaging cells (23) were transfected with MIY or MutY using a calcium phosphate coprecipitation method (24). After 24-hour culture, medium was replaced with DMEM supplemented with 20% FBS, and removed supernatant containing virus was cleansed by passage through a 0.20-μm syringe filter and frozen at –80°C until use. This procedure was repeated two more times every 24 hours.

Gene transfer and bone marrow transplantation. Three milligrams of 5-fluorouracil was intravenously injected into 8-week-old C57BL/6 mice. Three days later, bone marrow cells were collected from the femurs and tibiae and cultured on a 35-mm RetroNectin Dish (Takara Bio Inc., Shiga, Japan) with 3 ml of DMEM containing 20% FBS, 100 ng/ml stem cell factor, 100 ng/ml thrombopoietin, and 100 ng/ml Flt3-L (Genzyme Techne, Minneapolis, Minnesota, USA; Cosmo Bio Co., Tokyo, Japan) for 24 hours as prestimulation (25). The medium was then replaced by retrovirus-containing supernatant with addition of the same FBS and cytokine concentrations as those used in the prestimulation medium and was changed every day for 3 days. Then the cells on the 35-mm RetroNectin dish were harvested with cell dissociation buffer (GIBCO BRL; Invitrogen Corp., Carlsbad, California, USA) and injected into 8-week-old syngeneic recipients preconditioned with a single 9.0-Gy irradiation dose. For analysis of hematopoietic recon-
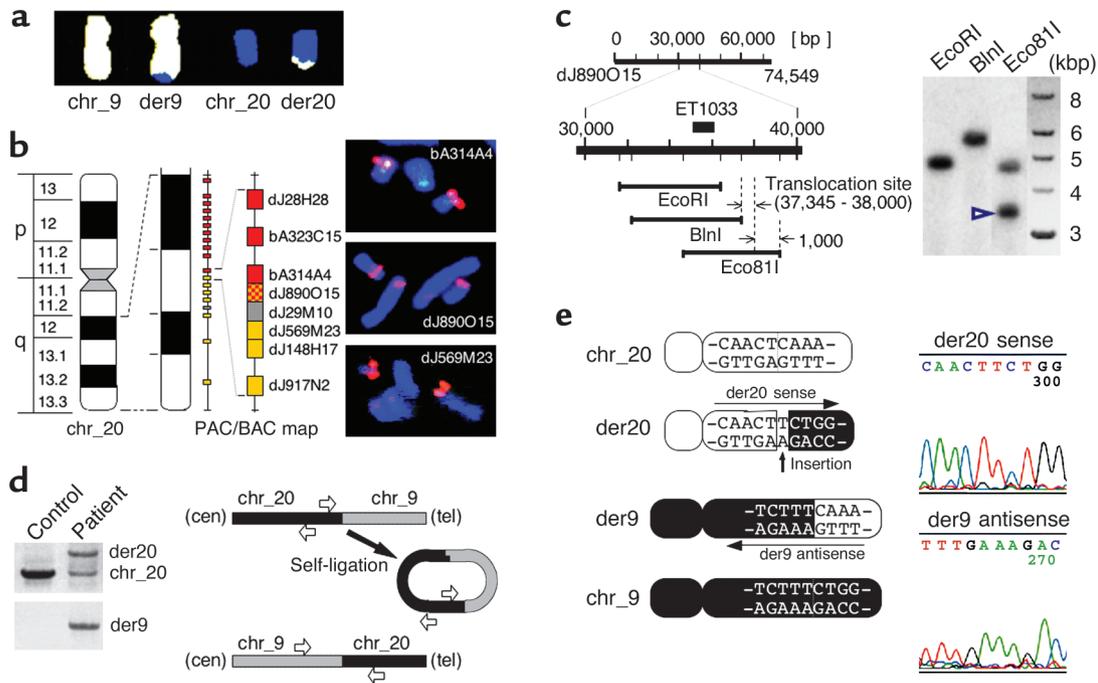
Figure 1

Detection of the translocation site. (**a**) Spectral karyotyping (SKY) FISH. (**b**) FISH. Red boxes indicate probes hybridized to chromosome 20 and der20; yellow boxes indicate probes hybridized to chr_20 and der9. PAC dJ890O15 was hybridized to chromosome 20, der20, and der9. (**c**) Genomic Southern blot hybridization with the ET1033 probe. The arrowhead indicates the mutation. (**d**) Genomic PCR products from chromosome 20, der20, and der9. cen, centromere; tel, telomere. (**e**) Sequences of the translocation site. Chr_9, chromosome 9; chr_20, chromosome 20.

stitution by flow cytometry, peripheral blood was obtained by retro-orbital sinus puncture, and bone marrow cells were obtained from femurs and tibiae 3 months after transplantation.

Primers. The following primers were used: ET1033s: 5′-GCACAGGTCTGTTATTTACCAGGTG-3′; ET1033a: 5′-CTGCCCCTTCTAGCCATTCTTTC-3′; NG1044: 5′-CAA-GAAGTACTCGTTTGAGTCGATCCGTGAG-3′; PH221: 5′-GTGTTGAACAGGTCCTCCTCCACCACCAAG-3′; NG459: 5′-GGAGCACTTTGTGTCTATCCTGCTGAAGTG-3′; TWIN2: 5′-TCAGCCTCCCAAGTAGCTGGGATTATAG-3′; NF9: 5′-GGTTGAACCATGATTCCGGTGACAGAGC-3′; AIN2: 5′-GAACAGAGCTTCTATCCTGGATGGTTCTG-3′
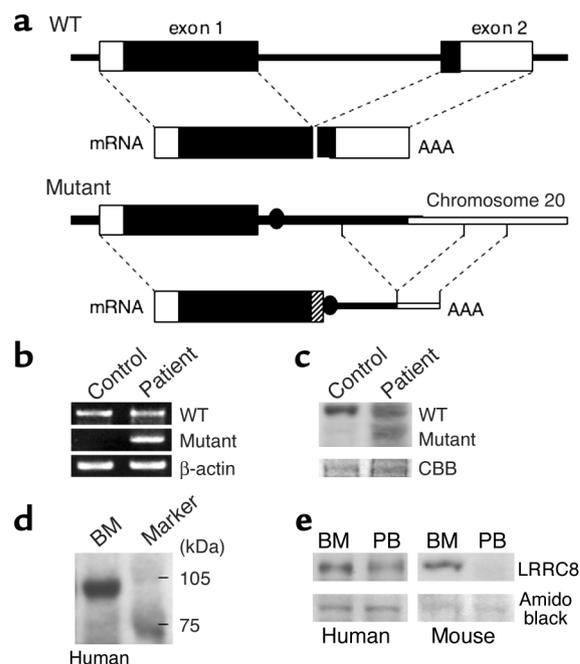
URLs. The following URLs were used: Contig map of the Sanger Centre, http://www.sanger.ac.uk/; GeneMap'99, http://www.ncbi.nlm.nih.gov:80/genemap/; GenBank, http://www.ncbi.nlm.nih.gov/Genbank/; TMHMM, http://www.cbs.dtu.dk/services/TMHMM/; Pfam, http://www.sanger.ac.uk/Software/Pfam/search.

shtml/; the Human Genome Organisation, http://www.gene.ucl.ac.uk/hugo/.

GenBank accession numbers. The following genes are mentioned in this paper: LRRC8, AY143166; dJ890O15, AL049540; RP11-98H23, AL136108; FLJ10337,

Figure 2

Products of LRRC8 and its mutant. (**a**) mRNAs of wild-type and mutant forms. Black boxes, coding regions; white boxes, untranslated regions; black circles, stop codons; hatched box, the translated region from the intron. (**b**) RT-PCR analysis. (**c–e**) Western blot analysis. (**c**) Peripheral blood cells from a healthy control and the affected patient. CBB, Coomassie brilliant blue. (**d**) Molecular weight estimation of the protein from healthy human bone marrow (BM). (**e**) Bone marrow and peripheral blood (PB) cells from a human control and a mouse.

a

Transmembrane 1
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Human 001 MIPVTELRYFADTQPAYRILKPWWDVFTDYISIVMLMIAVFGGTLQVTQDKMICLPCKWVTKDSCNDSFRGWAAPGPEPTYPNSTILPTPDTGPTGIKYD 100
Mouse 001 MIPVTELRYFADTQPAYRILKPWWDVFTDYISIVMLMIAVFGGTLQVTQDKMICLPCKWVTKDSCNDSFRGWAASSPEPTYPNSTVLPTPDTGPTGIKYD
```

Transmembrane 2
```
101 LDRHQYNYVDAVCYENRLHWFAKYFPYLVLLHTLIFLACSNFWFKFPRTSSKLEHFVSILLKCFDSPWTTRALSETVVEESDPKPAFSKMNGSMDKKSST
101 LDRHQYNYVDAVCYENRLHWFAKYFPYLVLLHTLIFLACSNFWFKFPRTSSKLEHFVSILLKCFDSPWTTRALSETVVEESDPKPAFSKMNGSMDKKSST
```

Transmembrane 3
```
201 VSEDVEATVPMLQRTKSRIEQGIVDRSETGVLDKKEGEQAKALFEKVKKFRTHVEEGDIVYRLYMRQTIIKVIKTLIICYTVYYVHNIKFDVDCTVDIE
201 VSEDVEATVPMLQRTKSRIEQGIVDRSETGVLDKKEGEQAKALFEKVKKFRTHVEEGDIVYRLYMRQTIIKVIKFALIICYTVYYVHNIKFDVDCTVDIE
```

Transmembrane 4
```
301 SLTGYRTYRCAHPLATLFKILASFYISLVIFYGLICMYTLWWMLRRSLKKYSFESIREESSYSDIPDVKNDFAFMLHLIDQYDPLYSKRFAVFLSEVSEN
301 SLTGYRTYRCAHPLATLFKILASFYISLVIFYGLICMYTLWWMLRRSLKKYSFESIREESSYSDIPDVKNDFAFMLHLIDQYDPLYSKRFAVFLSEVSEN
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```
                                                                                                LxxLxL
401 KLRQLNLNNEWTLDKLRQRLTKNAQDKLELHLFMLSGIPDTVFDLVELEVLKLELIPDVTIPPSIAQLTGLKELWLYHTAAKIEAPALAFLRENLRALHI
401 KLRQLNLNNEWTLDKLRQRLTKNAQDKLELHLFMLSGIPDTVFDLVELEVLKLELIPDVTIPPSIAQLTGLKELWLYHTAAKIEAPALAFLRENLRALHI
```

```
     xxNxLxxLPxxLxxL                                                                          LxxLxLxx
501 KFTDIKEIPLWIYSLKTLEELHLTGNLSAENNRYIVIDGLRELKRLKVLRLKSNLSKLPQVVTDVGVHLQKLSINNEGTKLIVLNSLKKMANLTELELIR
501 KFTDIKEIPLWIYSLKTLEELHLTGNLSAENNRYIVIDGLRELKRLKVLRLKSNLSKLPQVVTDVGVHLQKLSINNEGTKLIVLNSLKKVMNLTELELIR
```

```
     NxLxxLPxxLxxL   LxxLxLxxNxLxxLPxxL--xxL   LxxLxLxxNxLxxLPxxLxxL   LxxLxLxxNxLxxLPxxLxxL   LxxLxLxxNxLxxLxxL
601 CDLERIPHSIFSLHNLQEIDLKDNNLKTIEEIISFQHLHRLTCLKLWYNHIAYIPIQIGNLTNLERLYLNRRNKIEKIPTQLFYCRKLRYLDLSHNNLTFL
601 CDLERIPHSIFSLHNLQEIDLKDNNLKTIEEIISFQHVHRLTCLKLWYNHIAYIPIQIGNLTNLERLYLNRRNKIEKIPTQLFYCRKLRYLDLSHNNLTFL
```

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     PxxLxxL   LxxLxLxxNxLxxLPxxLxxL   LxxLxLxxNxLxxLPxxLxxL   LxxLxLxxNxLxxLPxxLxxL
701 PADIGLLQNLQNLAHTANRIEHLPPELFQCRKLRALHLGNNVLQSLPSRVGELTNLTQIELRGNRLECLPVELGECPLLKRSGLVVEEDLFNTLPPEVKE
701 PADIGLLQNLQNLAVTANRIEALPPELFQCRKLRALHLGNNVLQSLPSRVGELTNLTQIELRGNRLECLPVELGECPLLKRSGLVVEEDLFSTLPPEVKE
```

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801 RLWRADKEQA
801 RLWRADKEQA
```

c
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Mutant 001-700  same as the WT
       701-stop PADIGLLQNLQNLAITANRVSGPATALRVGWRVAWPGLVVGHSAGSVSWDRRCP
```

b

Figure 3
Structures of LRRC8 and its mutant. (**a**) Comparison of human LRRC8 and its murine ortholog. White letters on black boxes indicate different amino acids between the two species. LxxLxLxxNxLxxLPxxLxxL is the representative structure of LRR, where x means any amino acid. (**b**) Schematic structures. White boxes, LRRs; hatched boxes, transmembrane helices; black box, additional polypeptides. N, N-terminal; C, C-terminal. (**c**) Amino acid sequences of the mutant. Underlined characters represent translated polypeptides from the intron.
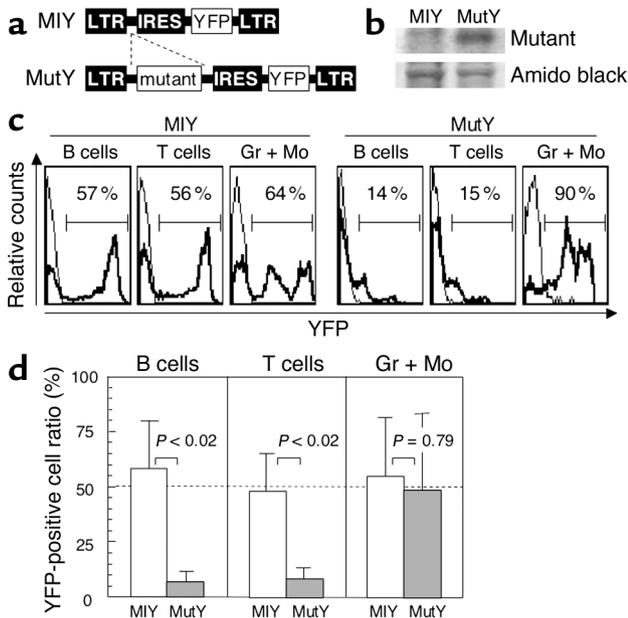
Results

Isolation of LRRC8 and its mutant form. Translocation between the long arms of chromosomes 9 and 20 was determined by spectral karyotyping (SKY) FISH analysis (Figure 1a). To identify the translocation site of the derivative of chromosome 20 (der20), we prepared BAC and PAC clones located on the long arm of chromosome 20. FISH analysis showed that the PAC clone dJ890O15 stepped over the translocation site (Figure 1b). According to Southern blot hybridization analysis with the ET1033 probe derived from dJ890O15, the translocation site was estimated to exist between the 37,345th and 38,000th bp of dJ890O15 (Figure 1c). We then analyzed the patient's DNA by inverse PCR and obtained PCR products containing the translocation site on der20 (Figure 1d). A cDNA fragment containing the translocation site on der9 was also obtained by PCR. Sequencing analysis of these products showed that the translocation site existed between the 122,214th and 122,215th bp of the BAC clone RP11-98H23 on chromosome 9, and between the 37,632nd and 37,633rd bp of the dJ890O15 on chromosome 20. A single nucleotide insertion (T) was observed at the translocation site on der20 (Figure 1e).

Although no genes located at the translocation site on chromosome 9 were found, exons or coding sequences for two full-length mRNAs, termed FLJ10337 and FLJ20996, and several spliced mRNAs were split and located over the translocation site, and computer analysis using both GENESCAN and HMMGENE predicted the existence of a hypothetical protein. There were no sequences for mRNA around the translocation site (~20 kbp) on chromosome 20. Sequences for the wild and mutant genes were obtained with 3′ rapid amplification of cDNA ends from peripheral white blood cells of the patient. Their schemata are shown in Figure 2a. Transcription from the mutant gene as well as the wild-type gene in the patient's cells was confirmed by RT-PCR (Figure 2b). Wild and mutant forms of the LRRC8 protein were also detected in the patient's cells by Western blot analysis (Figure 2c).

Characteristics of LRRC8. The length of the coding region of this gene was predicted to be 2,433 bp, and the protein consisted of 810 amino acids (~94.2 kDa). The result of Western blot analysis with poAb's against this protein was compatible with the predicted molecular weight (Figure 2d). It had two exons; exons 1 and 2 encoded 719 and 91 amino acids, respectively. The protein was produced at a higher level in bone marrow cells than in peripheral blood cells (Figure 2e).

granulocytes and monocytes of mice transplanted with MutY-infected bone marrow cells (Figure 4c). Statistical analysis is shown in Figure 4d. While our results show that expression of the truncated protein causes abnormalities in B and T cell development, they are consistent with the idea — but do not prove — that the mechanism by which this happens is dominant suppression by truncated proteins.

To examine the influence of the mutant protein on B cell development, B-lineage cell subsets in the bone marrow of the transplanted mice were analyzed with flow cytometry (Figure 5a). The development of B cells was arrested at the stage of pro-B (B220+, CD43+), and a severe reduction in the proportion of pre-B cells (B220+, CD43−) was observed. We examined protein expression on B-lineage cells belonging to different maturation stages (Figure 5b). Pro-B cells expressed the protein at a higher level than pre-B cells in the bone marrow, while the expression was much lower on peripheral B cells.

Discussion

We isolated a novel gene, *LRRC8*, from the translocation site on chromosome 9 of a patient with agammaglobulinemia, and this gene was truncated by the

Similarly, murine bone marrow cells contained greater amounts of the protein than peripheral blood cells.

The gene was highly conserved between humans and mice: 93% of nucleotide sequences and 99% of amino acid sequences were identical (Figure 3a). The structure of the protein predicted by TMHMM and Pfam computer-assisted analysis is shown in Figure 3b. It consisted of four transmembrane helixes located close to the N-terminal and nine leucine-rich repeats (LRRs) (26) close to the C-terminal. Both terminal ends were outside the cell, and the second to ninth LRRs were located consecutively. This gene was named LRRC8 by the nomenclature committee, the Human Genome Organisation. The truncated gene encoded 754 amino acids, including 719 derived from exon 1 and 35 from the intron (Figure 3c).

Both wild and mutant LRRC8 proteins were expressed on the patient's cells. There was no mutation in the coding sequences on the unaffected allele.
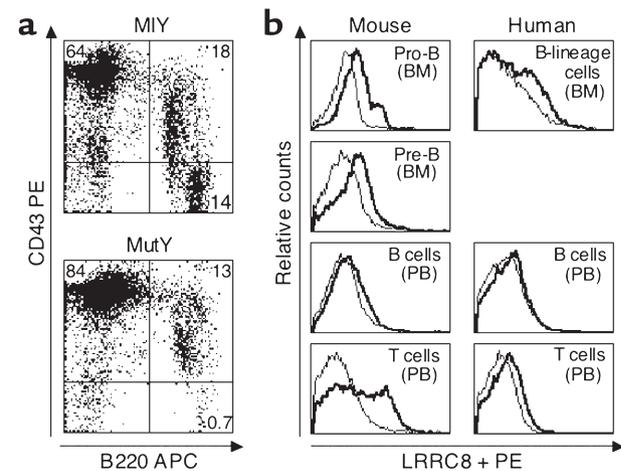
Significance of the mutant LRRC8. A mouse stem cell virus (MSCV) vector (27) including a fragment encoding yellow fluorescent protein (YFP) as a selection marker (MSCV-IRES-YFP), designated MIY (28), was prepared and used as a control vector (Figure 4a). Then, MutY was constructed with the mutant cDNA and MIY, which successfully yielded the mutant protein (Figure 4b) as well as YFP. To evaluate B cell reconstitution, lethally irradiated mice were transplanted with MIY- or MutY-infected bone marrow cells. The peripheral blood cells were analyzed by flow cytometry 3 months after transplantation, and the ratio of YFP-positive cells in each cell lineage was calculated. Although the efficiency of gene transfer into hematopoietic stem cells differed in each mouse, the ratio of YFP-positive cells in the B cell population was significantly decreased, and furthermore, that in the T cell population was decreased compared with those in

translocation. The patient's white blood cells expressed both wild-type and truncated LRRC8 proteins. LRRC8 is highly conserved between humans and mice: 93% and 99% of nucleotide and amino acid sequences were identical, respectively. Transplantation of bone marrow cells that were forced to express truncated LRRC8 to lethally irradiated mice induced a severe reduction of the B cell number in peripheral blood. Our results indicate that the *LRRC8* deficit is responsible for the B cell deficiency in this patient and that normal *LRRC8* is required for B cell development.

We showed that development of B-lineage cells expressing the truncated form of LRRC8 is arrested at the stage of pro-B, and the proportion of pre-B cells was severely reduced in the bone marrow. Since the highest expression of LRRC8 protein was seen on pro-B cells among subsets of B-lineage cells, this protein seems to be a key molecule for growth and/or differentiation in pro-B and pre-B cells.

The LRRs are functional domains that mediate adhesion to other molecules (26). The LRRs of LRRC8 are located on the outside of cells. Various molecules with such a structure are known to act as cell surface receptors, including RP105 (LPS receptor), CD42 (vWF receptor), TrkA (high-affinity nerve growth factor receptor), and follicle-stimulating hormone receptor (26, 29). It is possible that LRRC8 has a novel specific ligand that induces B cell development. LRRC8 lacks domains that can transduce signals to the cytosol or nucleus. RP105, a Toll-like receptor on B cells, has an adapter protein named MD-1 instead of a signal-transducing domain (30). Similarly, LRRC8 might also have adapter molecules for signal transduction.

The intact *LRRC8* gene on the unaffected allele of the patient was transcribed, and intact LRRC8 protein was expressed. Nevertheless, B cells were totally absent in the peripheral blood. This suggests that the truncated form has not a quantitative effect but a dominant-suppressor effect on B cell development. Autoimmune lymphoproliferative syndrome is ascribed to a dominant-negative mutant of Fas (31). Fas is a homotrimer of Fas subunits. When one of the three subunits is replaced with a mutant, the Fas complex loses its function. Therefore, only one-eighth of the receptors can transduce death signal into cells in patients with the heterozygous mutation. Dominant-negative mutants are known in several receptors that compose homooligomers: dynamin-1, growth hormone receptor, and insulin receptor (32–34). Although there are no known proteins containing LRR structures that form homooligomers, LRRC8 might form such a complex.

In addition to the deficiency of B cells, a decrease in the proportion of T cells among cells transfected with the mutant LRRC8 was also observed. Although both human and murine T cells express LRRC8 (data not shown), the number of T cells was not decreased in our patient. The difference between the function of LRRC8 in mice and its function in humans should be clarified in a further study.

Minor facial anomalies as well as agammaglobulinemia were observed in the patient. Using RT-PCR, we have determined that LRRC8 is expressed in brain, heart, lung, liver, and kidney (unpublished observations). Since this gene is expressed in a ubiquitous fashion beyond lymphoid and hematopoietic systems, the protein might therefore play a role in morphogenesis. A further analysis of *LRRC8* may contribute to our understanding of its roles in development.

There are no known proteins homologous to LRRC8, but there are at least two hypothetical proteins, AD158 and T cell activation leucine repeat–rich protein (TA-LRRP). There are also two predictable proteins, LRRC5 and FLJ23420, which may be fragments of other LRRC8 homologs. LRRC8, AD158, and TA-LRRP conserve 59% of their amino acid sequences and share a unique structure: four transmembrane helixes and eight sequentially located LRRs. TA-LRRP was found by a microarray assay of induced genes in activated T cells. These putative proteins, sharing a unique structure, constitute a novel protein family that has not, to our knowledge, been reported so far (unpublished observations). Although their functions remain to be elucidated, it seems plausible that they play essential and fundamental roles in morphogenesis.

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