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Identification and functional analysis of *CBLB* mutations in type 1 diabetes

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Abstract

Casitas B-lineage lymphoma b (Cblb) is a negative regulator of T-cell activation and dysfunction of Cblb in rats and mice results in autoimmunity. In particular, a nonsense mutation in *Cblb* has been identified in a rat model of autoimmune type 1 diabetes. To clarify the possible involvement of *CBLB* mutation in type 1 diabetes in humans, we performed mutation screening of *CBLB* and characterized functional properties of the mutations in Japanese subjects. Six missense mutations (A155V, F328L, N466D, K837R, T882A, and R968L) were identified in one diabetic subject each, excepting N466D. Of these mutations, F328L showed impaired suppression of T-cell activation and was a loss-of-function mutation. These data suggest that the F328L mutation is involved in the development of autoimmune diseases including type 1 diabetes, and also provide insight into the structure–function relationship of CBLB protein.

Keywords: Autoimmunity; *CBLB*; Genetics; Mutation; Susceptibility; T-cell; Type 1 diabetes

The majority of type 1 diabetes cases results from autoimmune destruction of the insulin-producing pancreatic beta-cells, and multiple genetic and environmental factors are involved. The major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) region, is the major genetic factor, but several other genes may be involved in the development of this disorder, such as *INS* (insulin), *CTLA4* (cytotoxic T lymphocyte antigen 4), *PTPN22* (protein tyrosine phosphatase non-receptor type 22), *IL2RA* (interleukin-2 receptor alpha, also known as *CD25*), *IFIH1* (interferon induced with helicase C domain 1, also known as *MDA5*), and four other chromosome regions (12q24, 12q13, 16p13, and 18p11) [1–3].

The Komeda diabetes-prone (KDP) rat has been established as a spontaneous animal model of type 1 diabetes, and is characterized by autoimmune destruction of pancreatic beta-cells, rapid onset of the disease with no sex difference, and no T-cell lymphopenia [4,5]. By positional

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cloning, we identified a nonsense mutation in Casitas B-lineage lymphoma b (*Cblb*) as a major factor in the disease in KDP rats [6]. Based on our previous studies [6,7], we proposed a two-gene model for the development of type 1 diabetes in KDP rats [8]. In this model, two major susceptibility genes, the MHC *RT1*^{u} haplotype and the *Cblb* mutation determine tissue specificity to pancreatic beta-cells and autoimmune reaction, respectively.

Cblb is a member of the RING type ubiquitin–protein ligases, and functions as a negative regulator of T-cell activation [9–11]. *Cblb* deficient mice exhibit several autoimmune reactions due to abnormal activation of T-cells [10,11]. Thus, Cblb is considered to be a negative regulator of autoimmunity, and dysfunction of CBLB may contribute to autoimmune diseases in humans, including type 1 diabetes.

Polymorphic variations of *CBLB* in human type 1 diabetes have been studied, but no association of the common variants (minor allele frequency [MAF] >0.03) with type 1 diabetes was observed [12,13]. By contrast, the exon 12 silent variant of *CBLB* (rs3772534, A621A, MAF = 0.02) was reported to be associated with type 1 diabetes in Danish subjects [14]. However, no association of this SNP with the disease was reported by the recent analysis using large family and case-control collections [15]. These studies suggest that the common variants of *CBLB* are not associated with human type 1 diabetes; however, association of rare variants with the disease remains to be further elucidated. In the present study, we performed mutation screening of *CBLB* in Japanese subjects with type 1 diabetes and characterized the functional properties of the missense mutations.

Materials and methods

Subjects. We examined 223 unrelated Japanese subjects with type 1 diabetes recruited from 6 university hospitals and affiliated hospitals located in 6 prefectures in Japan. Type 1 diabetes was diagnosed by both clinical features and laboratory data. All of the subjects were ketosis-prone, showed cessation of endogenous insulin secretion (C-peptide levels of <3.3 nmol/day), and needed more than four insulin injections per day. The clinical data on these type 1 diabetic subjects were as follows (continuous data was given as median with interquartile range): male, 33.3%; age at onset, 17 years (10–29 years). We also examined 192 non-diabetic control subjects matched for geographic region under the following criteria: 60 or more years of age, no past history of diagnosis of diabetes, HbA1c less than 5.6%, and no diabetes within third degree relatives. Genetic analysis of human subjects was approved by the ethics committee at each university or hospital. Appropriate informed consent was obtained from all of the subjects examined.

Mutation screening. Genomic DNA was extracted from peripheral blood samples by standard procedures. All of the exons and flanking intronic regions of *CBLB* were amplified by polymerase chain reaction (PCR) using LA *Taq* polymerase (TaKaRa, Tokyo, Japan). Primer sequences are available on request. The PCR products were purified using QIAquick spin column (Qiagen, Hilden, Germany) and sequenced by the dideoxy chain termination method using 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

HLA genotyping. HLA-*DRB1* and -*DQB1* alleles were determined by PCR-restriction fragment length polymorphism methods [16,17]. The most probable HLA-*DRB1*–*DQB1* haplotypes were deduced from known linkage disequilibrium [18].

Recombinant DNA construction. The NFAT luciferase reporter plasmid, which has an IL-2 minimal promoter with three tandem repeats of the NFAT binding site, was obtained from Dr. G. Crabtree (Stanford University). The full-length wild-type *CBLB* cDNA (GenBank Accession No. NM_170662) was cloned from the human spleen cDNA library (Multiple Choice cDNAs: Human Kit, OriGene Technologies, Inc., Rockville, MD, USA) by PCR using *Pyrobest* DNA polymerase (TaKaRa) and verified by DNA sequencing. The missense mutant *CBLB* cDNAs were generated by oligonucleotide-directed site-specific mutagenesis (Quick Change Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) and verified by DNA sequencing. All of the constructs were subcloned into the expression vector pEF1/His (Invitrogen, Carlsbad, CA, USA).

Cell culture and transfection. Jurkat-TAg (simian virus 40 T antigen) cells [19] were obtained from Dr. G. Crabtree (Stanford University). Jurkat-TAg cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum in a 5% CO_2, 95% air-humidified atmosphere. For transient transfections, 2 μg of the NFAT-luciferase (firefly luciferase) reporter plasmid and 0.6 μg of the *Renilla*-luciferase reporter plasmid were cotransfected with 4 μg of the control (pEF1/His-LacZ), wild-type, or mutant *CBLB* constructs into Jurkat-TAg cells (2×10^6 cells per well in 6-well tissue culture dishes) by the lipofection method (Lipofectamine 2000, Invitrogen).

Luciferase assay. The transfected cells were cultured for 24 h, and treated with 50 ng/ml PMA (Sigma–Aldrich, St. Louis, MO, USA) and 2 μg/ml αCD3 (BD Biosciences, San Jose, CA, USA). After 5 h, 75 μl of the cells was analyzed for luciferase activity (Dual-Glo Luciferase Assay System, Promega, Madison, WI, USA). Transfection efficiencies were normalized using *Renilla*-luciferase activity cotransfected with each experiment.

Statistical analysis. Differences in distribution of allele or genotype frequencies between type 1 diabetic subjects and control subjects were assessed using χ^2 tests or Fisher's exact probability tests. Differences in luciferase activities were assessed using two-tailed Student's *t* tests. Differences were considered statistically significant when $P < 0.05$.

Results

Mutation screening of CBLB in Japanese subjects with type 1 diabetes

To detect *CBLB* mutations in subjects with type 1 diabetes, we first screened all of the coding regions and exon-intron boundaries of the gene in 223 Japanese subjects with type 1 diabetes. We detected 26 variants, including 23 single nucleotide polymorphisms (SNPs) and three insertion/deletion variants of one or two nucleotides (Supplementary Table S1). Among the 12 exonic SNPs, six missense mutations (A155V, F328L, N466D, K837R, T882A, and R968L) were identified (Table 1 and Fig. 1A). We then examined 192 non-diabetic control subjects for these missense mutations. The N466D mutation was found in both diabetic and control subjects with similar allele frequencies. By contrast, the remaining five missense mutations (A155V, F328L, K837R, T882A, and R968L) were found in one diabetic subject each in the heterozygous state, and were not found in control subjects. As these missense mutations were rare, no statistically significant difference between them was shown in the case-control association analysis. In addition, while the exon 12 silent variant (rs3772534, A621A) was found frequently in both diabetic

Table 1
Frequency of missense mutations of *CBLB* in case and control subjects

Location	Mutation	Minor allele	Subject	Genotype data[a]			MAF	*P*-value
				1	2	3		
Exon 4	A155V (C/T)	T	Case	222	1	0	0.002	NS[b]
			Control	192	0	0	0	
Exon 7	F328L (T/C)	C	Case	222	1	0	0.002	NS
			Control	192	0	0	0	
Exon 10	N466D (A/G)	G	Case	214	9	0	0.020	NS
			Control	183	9	0	0.023	
Exon 12	A621A (G/A)[c]	A	Case	157	62	4	0.157	NS
			Control	142	49	1	0.133	
Exon 17	K837R (A/G)	G	Case	222	1	0	0.002	NS
			Control	192	0	0	0	
Exon 18	T882A (A/G)	G	Case	222	1	0	0.002	NS
			Control	192	0	0	0	
Exon 19	R968L (G/T)	T	Case	222	1	0	0.002	NS
			Control	192	0	0	0	

MAF, minor allele frequency; NS, non-significant.
[a] Genotype data are represented as 1, homozygote for the major allele; 2, heterozygote; and 3, homozygote for the minor allele.
[b] $P > 0.05$.
[c] The exon 12 silent variant is listed for reader's convenience.

B

	A155	F328	N466	K837	T882	R968
Human						
CBL	A	F	A	N	S	R
CBLB	A	F	N	K	T	R
CBLC	A	F	E	-	-	-
Cblb						
Chimpanzee	A	F	N	K	T	R
Cattle	A	F	N	K	S	R
Dog	A	F	N	K	S	R
Rat	A	F	S	K	S	R
Mouse	A	F	S	K	A	R
Chicken	A	F	S	K	T	R

Fig. 1. Missense mutations of CBLB and amino acid residues for the mutations in the CBL family of proteins. (A) Functional domains of the human CBLB protein and six missense mutations. CBLB protein consists of the tyrosine kinase binding region (TKB), RING finger domain (RING), proline-rich region (Pro-rich), and ubiquitin associated domain (UBA). The N-terminal TKB region is further divided into three structural domains, four helix bundles (4H), EF hand (EF), and SH2 domain (SH2). The positions of the six missense mutations identified in this study are indicated under the domain structure. (B) Amino acid residues for the mutations in the CBL family of proteins. Amino acid residues in the positions of the six missense mutations of the CBL family (CBL, CBLB, and CBLC) and Cblb orthologues of several species are listed. Note that CBLC lacks most of the C-terminal part of the protein including Pro-rich region and UBA domain.

and control subjects in this Japanese population, there was no statistically significant difference between them.

Most of the carriers of the six missense mutations possessed at least one HLA-DR4 (*DRB1*0405–DQB1*0401*) or -DR9 (*DRB1*0901–DQB1*0303*) haplotype (Supplementary Table S2), both of which are major type 1 diabetes susceptibility haplotypes in the Japanese population [20], which suggests that these subjects were typical Japanese patients with type 1 diabetes. Some of the carriers also had autoimmune thyroid diseases; the F328L carrier showed Hashimoto's thyroiditis, and one of the N466D carriers showed Graves' disease, an autoimmune thyroiditis.

The amino acid residues in the position of the three missense mutations (A155, F328, and R968) are well conserved not only in CBLB proteins of human, chimpanzee, cattle, dog, rat, mouse, and chicken, but in the human CBL family of proteins, CBL [21], CBLB, and CBLC [22], strongly suggesting its importance in function of the CBL protein family (Fig. 1B). K837 also is conserved in CBLB proteins of these seven species, while N466 and T882 are not.

Functional analysis of the six missense mutations of CBLB

To characterize the functional properties of the six missense mutations of CBLB (A155V, F328L, N466D, K837R, T882A, and R968L), the effect of expression of these mutant proteins on antigen receptor-mediated activation of the nuclear factor of activated T-cells (NFAT) promoter was evaluated by luciferase assay (Fig. 2A). The normalized relative luciferase activities were presented as a percentage of the activity of LacZ transfected cells (mean ± SEM, $N = 4$). When Jurkat-TAg cells were stimulated with anti-human CD3 monoclonal antibody (αCD3) and phorbol myristate acetate (PMA), NFAT transcription was significantly activated (αCD3 + PMA [−]: 2.4 ± 0.4% vs αCD3 + PMA [+]: 57.6 ± 5.6%; $P < 0.001$). When wild-type CBLB was expressed in Jurkat-TAg cells, NFAT

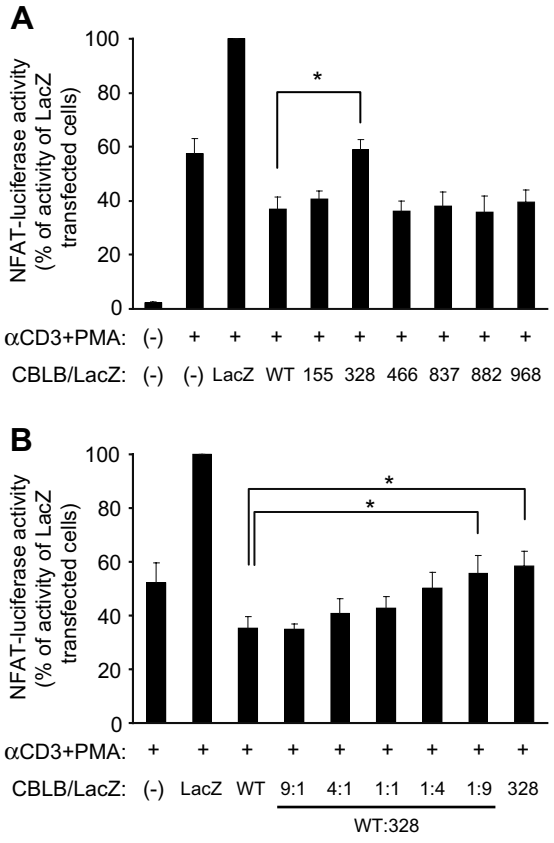

Fig. 2. Functional characterization of the missense mutations of CBLB. Effect of overexpression of the missense mutant proteins of CBLB on antigen receptor-mediated activation of NFAT promoter was evaluated in Jurkat-TAg cells. (A) Functional analysis of the six missense mutations of CBLB. Jurkat-TAg cells were transfected without (−) or with constructs encoding LacZ, wild-type (WT), or the six missense mutant proteins (155, 328, 466, 837, 882, or 968) in the presence of NFAT-luciferase and *Renilla*-luciferase reporter plasmids. $^{*}P < 0.01$ vs WT. (B) Functional analysis of the F328L missense mutation of CBLB. Jurkat-TAg cells were transfected without (−) or with constructs encoding LacZ, wild-type (WT), F328L (328), or varying combinations of WT and F328L (WT:328; 3.6 μg:0.4 μg, 9:1; 3.2 μg:0.8 μg, 4:1; 2 μg:2 μg, 1:1; 0.8 μg:3.2 μg, 1:4; 0.4 μg:3.6 μg, 1:9) in the presence of NFAT-luciferase and *Renilla*-luciferase reporter plasmids. $^{*}P < 0.05$ vs WT.

activation was significantly suppressed (LacZ: 100% vs WT: $37.0 \pm 4.4\%$; $P < 0.001$). Similar to wild-type CBLB, five of the six mutant proteins (A155V, N466D, K837R, T882A, and R968L) suppressed NFAT activation (five mutants vs WT; $P > 0.05$). By contrast, the F328L mutant protein showed impaired suppression compared with wild-type CBLB (F328L: $59.0 \pm 3.6\%$ vs WT: $37.0 \pm 4.4\%$; $P = 0.008$). The expressions of these mutant and wild-type CBLB proteins were confirmed by western blot analysis (data not shown). These results indicate that the CBLB F328L mutant protein has impaired function in the regulation of T-cell activation.

F328L is a loss-of-function mutation

To further characterize the functional properties of the CBLB F328L mutant protein, we performed NFAT activation assay with varying combinations of F328L with wild-type CBLB (Fig. 2B). The normalized relative luciferase activities were presented as a percentage of the activity of LacZ transfected cells (mean ± SEM, $N = 4$). When varying amounts of the F328L mutant protein were expressed together with varying amounts of wild-type CBLB, wild-type CBLB showed a dose-dependent suppression of NFAT activation and the F328L mutant protein did not exert a dominant-negative effect (9:1 [$34.9 \pm 1.9\%$], 4:1 [$40.8 \pm 5.3\%$], 1:1 [$42.8 \pm 4.3\%$], 1:4 [$50.3 \pm 5.8\%$], 1:9 [$55.6 \pm 6.9\%$], or F328L [$58.5 \pm 5.4\%$] vs WT [$35.3 \pm 4.3\%$]; non-significant [NS], NS, NS, NS, $P = 0.047$, or $P = 0.015$, respectively). The expressions of the F328L mutant and wild-type CBLB proteins were confirmed by western blot analysis (data not shown). These data indicate that F328L is not a dominant-negative but rather a loss-of-function mutation.

Discussion

Our previous studies using KDP rats [6], together with studies of *Cblb* deficient mice [10,11], strongly suggest that *CBLB* is a potential candidate for autoimmune diseases in humans, including type 1 diabetes. Although common variants of *CBLB* seem not to be associated with type 1 diabetes [12–15], it is yet possible that rare variants are involved in the development of the disease. In the present study, we found six missense mutations (A155V, F328L, N466D, K837R, T882A, and R968L) of *CBLB* in Japanese subjects with type 1 diabetes. The N466D mutation was detected in both diabetic and control subjects (MAF = 0.02), while the remaining five mutations were rare (MAF = 0.002) and were found in one diabetic subject each in the heterozygous state, suggesting that *CBLB* mutations are rare in Japanese subjects with type 1 diabetes. However, the F328L mutation showed impaired function in the regulation of T-cell activation, and is most probably a loss-of-function mutation, suggesting that F328L is involved in the development of autoimmune diseases including type 1 diabetes.

This study is the first detailed screening of *CBLB* with functional characterization of the mutations in relation to type 1 diabetes. For a genetic association study, the 223 case and 192 control subjects used in the present study were too few. Therefore, we focused on screening for mutations that might be associated with the disease, and identified several mutations including one loss-of-function mutation.

F328 is located in the SH2 domain of the N-terminal region of CBLB protein (Fig. 1A). The phenylalanine residue is highly conserved in the CBL family of proteins (Fig. 1B). The N-terminal tyrosine kinase binding (TKB) region comprising three structural domains (four helix bundles, EF hand, and SH2 domain) is evolutionarily conserved. Our functional characterization indicates that F328L is a loss-of-function mutation, suggesting that the mutant protein could have impaired binding capacity

and substrate specificity. However, since the binding capacity and substrate specificity are not determined by the TKB region alone [23], the mechanism of impaired suppression of T-cell activation by the F328L mutant protein is yet unclear.

Although the other missense mutations showed no significant difference in the regulation of T-cell activation compared to wild-type in the present assay system, the R968L mutation is interesting because it is located in the ubiquitin associated (UBA) domain of the C-terminal region of CBLB protein (Fig. 1A). In addition, the arginine residue is evolutionarily conserved in the CBL family of proteins (Fig. 1B). Since the UBA domain of Cblb is involved in both binding with ubiquitin and homodimerization with Cblb or heterodimerization with Cbl [24], the R968L mutation might well impair function.

In our recent study, autoimmune thyroiditis was frequently observed in the *Cblb*-congenic rat strain, TM.KDP-*Cblb* [25]. Thus, the *CBLB* gene also is a potential candidate for autoimmune thyroid diseases. It is noteworthy that the carrier of F328L mutation exhibited type 1 diabetes and Hashimoto's thyroiditis, and that her daughter did not have type 1 diabetes but had Hashimoto's thyroiditis (Supplementary Table S2). While it remains to be clarified whether the F328L mutation was transmitted in the family, the family history suggests the possible involvement of the mutation in autoimmune diseases.

In conclusion, we identified six missense mutations of *CBLB* in Japanese subjects with type 1 diabetes, and found that the F328L mutation is most likely a loss-of-function mutation. These findings suggest that the F328L mutation of *CBLB* is associated with autoimmune diseases including type 1 diabetes. Further characterization of the missense mutations of *CBLB* should provide insight into the structure-function relationship of this protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.032.

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