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Alternative Splicing Forms of the Human CD1D Gene in Mononuclear Cells

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CD1d is a critical molecule for the presentation of lipid antigens to natural killer (NK) T cells. To investigate the molecular complexity of CD1d, alternatively spliced transcripts in peripheral blood mononuclear cells from three healthy subjects were analyzed by PCR and sequencing methods. We found eight alternatively spliced variants of the CD1D gene (V1–V8), seven of which are newly established variants (V2–V8). V1 and V4 are in-frame; however, the other six variants (V2, V3, V5–V8) are out-of-frame. V1, V2, V4, and V5 lack a β_2-microglobulin binding site (α3 domain), indicating the unstable presentation of the CD1d molecule on the surface. In V2 and V5, the transmembrane region is absent, supporting a soluble CD1d. In the V3–V8 variants, the antigen binding region (α1 and α2 domains) is partially defective, suggesting incomplete functional products. In contrast, the V1 and V2 transcripts bear the complete antigen binding site, resulting in functional proteins. Especially, the V2 splicing variant might function as an inhibitory soluble CD1d molecule and regulate the presentation of antigens on APC to NKT cells.

Key Words: **alternative splicing variant; CD1d; CD1D gene; NKT cells; soluble CD1d.**

Human CD1 is a nonpolymorphic major histocompatibility complex (MHC) class I-like molecule (1–3). This glycoprotein is composed of a 43- to 49-kDa heavy chain in noncovalent association with a 12-kDa β_2-microglobulin (β_2-m) light chain. CD1 antigens are expressed at the surface of cortical thymocytes (4), B cells (5–7), dendritic cells (7–9), Langerhans cells in the skin (10), and gastrointestinal epithelial cells (7, 9). Their function is to present lipid antigens to T cells expressing $\alpha\beta$ or $\gamma\delta$ T cells including NKT cells (11–15). The CD1 genes map to chromosome 1q22-23 (16). Five CD1 genes (CD1A, CD1B, CD1C, CD1D, and CD1E) have so far been identified (1, 17, 18). CD1 proteins are classified into two groups based on comparison of their sequences (19). Group1 consists of CD1a, CD1b, and CD1c, and these molecules are expressed on professional antigen presenting cells. Group 2 comprises the CD1d protein, and this is mainly expressed on dendritic cells or epithelial cells of the gastrointestinal tract (7, 9).

Recently it has been demonstrated that CD1d molecules are able to present glycolipids to natural killer (NK) T cells (20, 21). NK T cells express the antigen receptor of T cells, T cell receptor (TCR), and NK cell surface marker, NKR-P1A (CD161). This population utilizes a unique TCRα chain, an invariant TCR AV24AJ18 gene without an N insertion in the N region. There are several reports that NKT cells may function as regulatory T cells and that a decrease in the number of NKT cells is associated with the pathogenesis of autoimmune diseases (15, 22–26). However, the mechanism of the selective reduction of NKT cells in autoimmune diseases has not been clarified. Thus, to investigate the molecular complexity of CD1d, alternative spliced variants, including a soluble CD1d molecule, were analyzed in peripheral blood mononuclear cells from healthy subjects. We established seven alternative splicing variants of the CD1D gene (V2–V8); the V2 variant is a soluble form with an antigen binding site. The cause of the reduction of NKT cells in autoimmune diseases is also discussed.

MATERIALS AND METHODS

RNA preparation and PCR. PBMC in 10 ml of heparinized peripheral blood from three healthy subjects were isolated by Ficoll-Paque separation (Pharmacia Biotech Inc., Piscataway, NJ). Total RNA was prepared from fresh PBMC with Isogen (Nippon Gene, Co., Tokyo, Japan), and reverse transcribed into complementary DNA (cDNA) using methods described elsewhere (27). Briefly, first-strand cDNA was synthesized in a 20 μl reaction mixture containing oligo(dT) primer from 1 μg of total RNA. A 0.2-μl aliquot of the reaction mixture encoding the cDNA was used for first-round PCR analysis in 50 μl of standard buffer containing 100 μM each of primers specific for the human CD1D 5′-untranslated region (positions 106–126)

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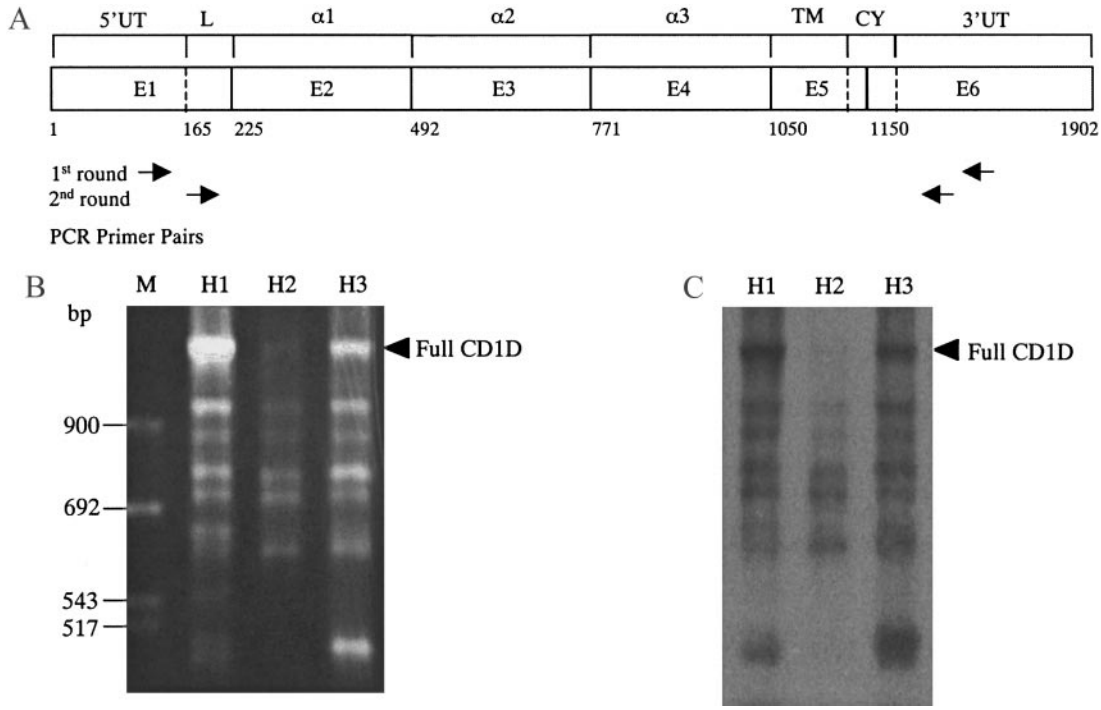

FIG. 1. Diagrammatic representation of the full human CD1D transcript and the primer sites for the nested RT-PCR. (A) The CD1D transcript comprises six exons (E1–E6) encoding the leader (L), α1, α2, α3 domains, transmembrane (TM), and cytoplasmic (CY) domains. (B) RT-PCR analysis of the CD1D gene. PCR products were analyzed on agarose gels by ethidium bromide staining. M, size markers; H1–H3, three healthy subjects. (C) Southern blot analysis of amplified PCR products encoding the CD1D gene.

(5′-AGAAGAGTGCGCAAGTCAGAG-3′) and the CD1D 3′-untranslated region (positions 1184–1205) (5′-TGGGTTCCAGAGACACAG-ATG-3′), and 1.25U of *Taq* polymerase (5 U/μl) (Takara Shuzo, Co., Ltd., Shiga, Japan). The denaturing step was carried out at 94°C for 1 min, the annealing step at 55°C for 1 min, and the extension step at 72°C for 1 min for 30 cycles on a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT). Two-microliter aliquots of the first-round PCR were used for second-round PCR, carried out using fully nested primers specific for the CD1D leading region (positions 165–186: 5′-primer: 5′-ATGGGGTGCCTGCTGTTTCTG-3′), and CD1D cytoplasmic region (positions 1161–1178: 3′-primer: 5′-GGCGAGT-CACAGGACGCC-3′) under the same PCR conditions described above. Aliquots of PCR products were subjected to gel electrophoresis and visualized by ethidium bromide staining. Primers for the detection of the CD1D gene are shown in Fig. 1A.

Southern blot analysis. Amplified DNAs were transferred to Immmobilon-S (Millipore Intertech, Bedford, MA) and hybridized with biotinylated CD1D probes, streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Millipore Intertech.

Sequencing of cDNAs encoding CD1D genes. For cloning and sequencing, products encoding the CD1d genes amplified by PCR were purified with a QIAauick PCR Purification kit (QIAGEN, Hiden, Germany), and were randomly cloned into pCR2.1 vector (Invitrogen, San Diego, CA). The nucleotide sequences of the cloned genes were analyzed with an ABI377 sequencer (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

To examine whether variant splicing forms exist in PBMC, DNAs encoding the CD1D gene were amplified by nested PCR with primers specific for CD1D (Fig. 1A). As shown in Figs. 1B and 1C, at least seven bands encoding the CD1D gene were detected by ethidium bromide staining and confirmed by Southern blot analysis. To determine whether the amplified DNAs are splicing variants of the CD1D gene or not, the nucleotide sequences of the PCR products were determined. We obtained evidence that there were eight alternative splicing forms (V1 to V8) in addition to the full CD1D gene in PBMC from all three healthy subjects. The eight variants and one entire CD1D transcript are summarized in Fig. 2. The V1 variant is the same splicing variant form as the exon 4-deficient CD1D transcripts in human choriocarcinoma cell lines (28). This is the first report of the other seven variant transcripts. Two spliced variant forms, V1 and V4, are in-frame. The other six forms are out-of-frame.

V2 and V5 are defective in the transmembrane region, resulting in soluble or intra-cellular proteins. A previous report of HLA-G molecules lacking the transmembrane region showed soluble forms (29, 30). Woolfson *et al.* (31) demonstrated that the CD1A, C, and E genes represent mRNA splicing variants, including those that lack a membrane attachment site. They also showed that splicing variants of the CD1A and CD1C transcripts include both secretary and intracellular forms. Thus, it is possible that CD1D transcript

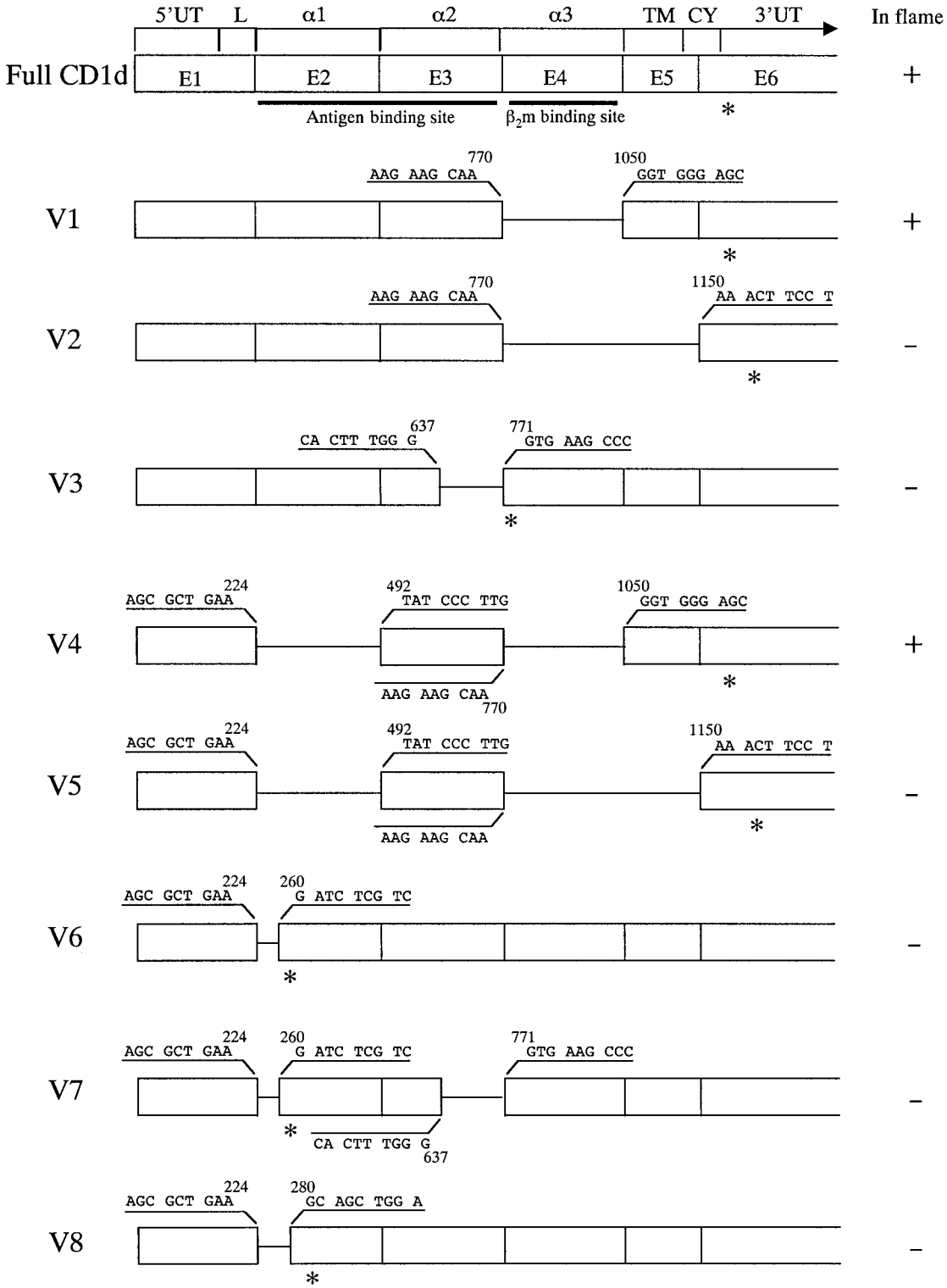

FIG. 2. Structures of alternatively spliced CD1D transcripts. The scheme at the top of the figure illustrates the exon organization of the CD1D gene. Exons 2 to 4 are the α1–α3 extracellular domains, E2 and E3 construct the antigen binding site, and E4 is the β2-m binding site. V1 to V8 are eight alternatively spliced forms of CD1D transcripts. Numbers indicate the nucleotide positions in the CD1D gene. *, stop codon; +, in-frame; −, out-of-frame.

variants lacking a transmembrane region represent both forms, although experiments involving ELISA or immunoprecipitation are necessary to confirm the existence of secretary or intra-cellular forms.

V1, V2, V4, and V5 have no α3 domain encoding the β_2-m binding site, suggesting unstable presentation of the antigen (32, 33). The antigen binding region is deleted partially in the V3 to V8 splicing variants, indicating that these molecules are not able to bind antigens presented by conventional CD1d. Thus, these six variants (V3–V8) may be incomplete functional form, although it can not be excluded the possibility to present unknown antigen. In contrast, the V1 and V2 variants contain the complete antigen binding region, suggesting that antigen binding capacity is retained. Although V1 lacks the α3 domain and can not bind to β_2-m, this form is in-frame and presented on the cell surface because CD1d can be transported to the cell surface independent of β_2-m (34, 35). The β_2-m-independent form of CD1d shows altered immature glycosylation (35), and the V1 form may contain an immature glycoprotein, suggesting the inadequate presentation of antigen for the majority of T cells. Therefore, the V1 form might function as an inhibitory molecule for antigen presentation.

The V2 variant conserves the intact antigen-binding site, although it lacks both the β_2-m binding domain and the transmembrane region. Therefore, this soluble variant may act as a competitive inhibitor of the CD1d molecule. Measurements of the V2 variant in the serum of patients with autoimmune diseases may help to clarify the mechanism of the decrease in NKT cells.

In human autoimmune diseases such as systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and insulin dependent diabetes mellitus, the numbers of NKT cells are selectively reduced (22–25), suggesting that these cells are regulatory T cells. Recently, our study (Kojo *et al.*, submitted) suggested that the decrease in the number of NKT cells in autoimmune diseases is due to an inadequate ligand, NKT cell dysfunction, or a lower presentation of antigen on APC. Alternative splicing transcripts, such as V1, might be one reason for the lower presentation of antigen on APC variants in patients with autoimmune diseases. In addition, the soluble V2 variant may regulate the interaction between NKT cells and the CD1d molecule on APC, causing the reduction in the number of NKT cells. Elucidation of the expression of the CD1d molecule and the ratio of the V1 to V2 transcripts should shed light on the mechanism of autoimmunity.

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