Naruse TK, Okuda Y, Mori K, Akari H, Matano T, Kimura A (2011) ULBP4/RAET1E is highly polymorphic in the Old World Monkey. Immunogenetics 63:501–509

Otting N, Otting N, de Vos-Rouweler AJM, Heijmans CMC, de Groot NG, Doxiadis GGM, Bontrop RE (2007) MHC class I A region diversity and polymorphism in macaque species. Immunogentics 59:367–375

Pappworth IY, Wang EC, Rowe M (2007) The switch from latent to productive infection in Epstein-Barr virus-infected B cell is associated with sensitization to NK cell killing. J Virol 81:474–482

Pende D, Rivera P, Marcenaro S, Chang CC, Biassoni R, Conte R, Kubin M, Cosman D, Ferrone S, Moretta L, Moretta A (2002) Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res 62:6178–6186

Radaev S, Rostro B, Brooks AG, Colonna M, Sun PD (2001) Conformational plasticity revealed by the cocrystal structure of NKG2D and its class I MHC-like ligand ULBP3. Immunity 5: 1039–1049

Radosavljevic M, Cuillerier B, Wilson MJ, Clement O, Wicker S, Gilfillan S, Beck S, Trowsdale J, Bahram S (2001) A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-q25.3. Genomics 79:114–123

Raulet DH (2003) Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 3:781–790

Richard J, Phan TNQ, Ishizuka Y, Cohen EA (2013) Viral protein R upregulates expression of ULBP2 on uninfected bystander cells during HIV-1 infection of primary CD4+ T lymphocytes. Virology 443:248–256

Romphruk AV, Romphruk A, Naruse TK, Raroengjai S, Puapairoj C, Inoko H, Leelayuwat C (2009) Polymorphisms of NKG2Dligands: Diverse RAET1/ULBP genes in northeastern Thais. Immunogenetics 61:611–617

Saito Y, Naruse TK, Akari H, Matano T, Kimura A (2012) Diversity of MHC class I haplotypes in cynomolgus macaques. Immunogenetics 64:131–141

Spreu J, Stehle T, Steinle A (2006) Human cytomegalovirus-encoded UL16 discriminates MIC molecules by their α2 domains. J Immunol 177:3143–3149

Ward J, Bonaparte M, Sacks J, Guterman J, Fogli M, Mavilio D, Barker E (2007) HIV modulates the expression of ligands important in triggering natural killer cell cytotoxic responses on infected primary T-cell blasts. Blood 110:1207–1214

Ward J, Davis Z, DeHart J, Zimmerman E, Bosque A, Brunette E, Mavilio D, Placeless V, Barker E (2009) HIV-1 PR triggers natural killer cell-mediated lysis of infected cells through activation of the ATR-mediated DNA damage response. PLoS Pathogen 5:e1000613

Wittenbrink M, Spreu J, Steinle A (2009) Differential NKG2D binding to highly related human NKG2D ligands ULBP2 and RAET1G is determined by a single amino acid in the α2 domain. Eur J Immunol 39:1642–1651

Wu J, Song Y, Bakker ABH, Bauer S, Spies T, Lanier LL, Phillips JH (1999) An activating immunoreceptor complex formed by NKG2D and DAP 10. Science 285:730–732

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A Naturally Occurring Single Amino Acid Substitution in Human TRIM5α Linker Region Affects Its Anti-HIV Type 1 Activity and Susceptibility to HIV Type 1 Infection

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Abstract

TRIM5α is a factor contributing to intracellular defense mechanisms against retrovirus infection. Rhesus and cynomolgus monkey TRIM5αs potently restrict HIV-1, whereas human TRIM5α shows weak effects against HIV-1. We investigated the association between a single nucleotide polymorphism in the TRIM5α linker 2 region (rs11038628), which substituted aspartic acid (D) for glycine (G) at position 249, with susceptibility to HIV-1 infection in Japanese and Indian subjects. rs11038628 is rare in Europeans but common in Asians and Africans. Functional analyses were performed by multiple-round replication and single-round assays, and indicated that the G249D substitution attenuated anti-HIV-1 activity of human TRIM5α. A slight attenuation of anti-HIV-2 activity was also observed in TRIM5α with 249D. The predicted secondary structure of the linker region suggested that the 249D substitution extended the α-helix in the neighboring coiled-coil domain, suggesting that human TRIM5α with 249D may lose the flexibility required for optimal recognition of retroviral capsid protein. We further analyzed the frequency of G249D in Japanese (93 HIV-1-infected subjects and 279 controls) and Indians (227 HIV-1-infected subjects and 280 controls). The frequency of 249D was significantly higher among HIV-1-infected Indian subjects than in ethnicity-matched control subjects [odds ratio (OR) = 1.52, $p = 0.026$]. A similar weak tendency was observed in Japanese subjects, but it was not statistically significant (OR = 1.19, $p = 0.302$). In conclusion, G249D, a common variant of human TRIM5α in Asians and Africans, is associated with increased susceptibility to HIV-1 infection.

Introduction

TRIM5α FROM RHESUS MONKEYS restricts human immunodeficiency virus-1 (HIV-1) replication at the postentry,[1] preintegration stage in the viral life cycle through rapid degradation of the HIV-1 core,[2] whereas human TRIM5α restricts HIV-1 only weakly but potently restricts N-tropic murine leukemia virus.[3,4] TRIM5α is a member of the tripartite motif-containing proteins and consists of RING, B-box 2, coiled-coil, and PRYSPRY (B30.2) domains. TRIM5α recognizes the multimerized capsid (CA) proteins of an incoming virus by its α-isoform-specific PRYSPRY domain. Studies of chimeric TRIM5αs have shown that the determinant of species-specific restriction against viral infection resides in the variable regions of the PRYSPRY domain.[5–11]

Infection by HIV-1 and progression to acquired immune deficiency syndrome (AIDS) vary among human individuals, and these phenomena are considered to be at least partially controlled by diversity in the human genome.[12,13] Two common TRIM5α functional polymorphisms, H43Y and R136Q, have been studied with regard to the association with HIV-1 infection.[14–21] Price *et al.* sequenced exon 2 of the *TRIM5* gene in 1,032 women enrolled in a long-term monitored Pumwani sex worker cohort, and found that women with the R136Q polymorphism were less likely to seroconvert despite heavy exposure to HIV-1 through active sex work.[15] Previous studies, including ours, showed the reduced antiviral activity of the H43Y substitution, but the associations with HIV-1 infection and disease progression were inconsistent among studies.[14,16–20] Javanbakht *et al.* reported a paradoxical

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protective effect of TRIM5α with 43Y against HIV-1 transmission in African-Americans.[14] Taken together, these findings indicate that anti-HIV-1 activity of human TRIM5α cannot protect humans from an HIV-1 pandemic, but may affect the rate of HIV-1 transmission.

In the present study, we investigated the association between a single nucleotide polymorphism (SNP) in the TRIM5α linker 2 region (rs11038628) between coiled-coil and PRYSPRY domains with susceptibility to HIV-1 infection. This SNP substituted aspartic acid (D) for glycine (G) at position 249. We show here that this SNP is associated with increased susceptibility to HIV-1 infection.

Materials and Methods

Cloning and expression of TRIM5α

The generation of recombinant Sendai viruses (SeVs) expressing human TRIM5α derived from MT4 cells, rhesus monkey TRIM5α derived from LLC-MK2 cells, and cynomolgus monkey TRIM5α lacking the PRYSPRY domain has been previously described.[9,22] All these TRIM5αs carried a hemagglutinin (HA) tag (YPYDVPDYAA) at the C-terminus. The D-to-G substitution at the 249th position was introduced into MT4 TRIM5α by polymerase chain reaction (PCR) site-directed mutagenesis. The resultant PCR fragment was cloned into pSeV18+b(+) as a vector. Recombinant SeVs expressing human TRIM5α carrying G at position 249 were recovered according to the previously described method.[23] The second passages in embryonated chicken eggs were used as stock virus for all experiments.

Western blotting analysis

MT4 cells (1×10^6) infected with recombinant SeVs expressing HA-tagged TRIM5α proteins were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). TRIM5α proteins in the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were then electronically transferred onto a membrane (Immobilon; Millipore, Billerica, MA). Blots were blocked and probed with anti-HA high-affinity rat monoclonal antibody (Roche, Indianapolis, IN) overnight at 4°C. Blots were then incubated with peroxidase-conjugated anti-rat IgG (American Qualex, San Clemente, CA), and bound antibodies were visualized with a Chemilumi-One chemiluminescent kit (Nacalai Tesque, Kyoto, Japan).

Viral infection

MT4 cells (1×10^6) were infected with SeVs expressing MT4-derived human TRIM5α (249D), human TRIM5α (249G), rhesus monkey TRIM5α, or cynomolgus monkey TRIM5α lacking the PRYSPRY domain [CM-TRIM5α-SPRY(−)] at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell and incubated at 37°C for 9 h. Aliquots of 1×10^5 cells were then superinfected with HIV-1 NL43 or HIV-2 GH123. Each superinfection used a titer of virus corresponding to 7 ng of p24 of NL43 or 20 ng of p25 of GH123. Experiments were performed with triplicate samples. The culture supernatants were collected periodically and the level of p24 or p25 was measured using a RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, NY). For the single-round infection assay, hamster TK-ts13 cells were infected with SeV expressing TRIM5α as described above, and superinfected with a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 vector expressing green fluorescence protein (GFP) under the control of the cytomegalovirus (CMV) promoter. The original HIV-1 vector was based on the BH10 strain.[24,25] To construct the lentivector possessing CA of NL4-3, we replaced the *Eco*RI–*Apa*I fragment corresponding to MA and CA of the pMDLg/p.RRE packaging vector with that of NL4-3.[26] In case of HIV-2, we used a canine cell line Cf2Th and VSV-G pseudotyped HIV-2 vector expressing GFP under the control of the LTR promoter.[27] Two days after infection, the cells were fixed with formaldehyde, and GFP-expressing cells were counted by a flow cytometer.

Human DNA subjects

The protocol for the present study was approved by the Ethics Review Board of the Medical Research Institute of Tokyo Medical and Dental University and that of the All India Institute of Medical Science. At setup of the cohort of HIV-1-infected Japanese subjects with hemophilia in 1995, all patients had been infected for longer than 10 years but were asymptomatic without any antiviral measures. Blood samples were collected from 93 well-characterized patients who were selected from the cohort after obtaining written informed consent.[28,29] Control DNA samples were prepared from Epstein–Barr virus-transformed human B cell lines established from randomly selected healthy donors ($n=279$) and obtained from the Japan Health Sciences Foundation. DNA samples from HIV-1-infected individuals were prepared from the blood samples using a QuickGene DNA whole blood kit S (Fujifilm, Tokyo, Japan). In addition, blood DNA samples were obtained from 227 HIV-1-infected Indian subjects and 226 healthy Indian volunteers with informed consent in related hospitals with the All India Institute of Medical Sciences, New Delhi.

Identification and genotyping of nucleotide variations in TRIM5α exon 5

Primer sets were designed to amplify the genomic segments covering the entire TRIM5α exon 5 as follows: sense primer (5′-GATGCGGTCATGCTATGTTG-3′) and antisense primer (5′-CGAATGCTGATTTATGACCATA-3′). Genomic DNA was subjected to PCR amplification followed by sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Polymorphisms were identified using the Sequencher program (Gene Code Co., Ann Arbor, MI).

Statistical analysis

All statistical analyses in this study were performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA). Pairwise linkage disequilibrium (LD) (r^2) was estimated using SNPAlyze version 6.0 standard (Dynacom Co., Ltd., Chiba, Japan).

Prediction of the peptide secondary structure

The Chou–Fasman methods were used to predict the secondary structure of TRIM5α using GENETYX-MAC version 15 software (Genetyx Corporation, Tokyo, Japan).

Results

Anti-HIV-1 activity of TRIM5α was attenuated by G249D substitution

We previously cloned human TRIM5α from the CD4-positive T cell line MT4 and noted that there is a G-to-D amino acid substitution (G249D) in comparison with the reference sequence (NM_033034).[9] This position is known as a polymorphic site in the human *TRIM5* gene (rs11038628) located in the linker 2 region between the coiled-coil and PRYSPRY domains (Fig. 1). Initially, we speculated that this polymorphism would have no effect on antiviral activity due to its presence in the linker 2 region. Goldschmidt *et al.*[18] reported that HeLa cells stably transduced with TRIM5α with 249D did not differ in susceptibility to HIV-1 infection. However, a tendency toward higher *in vitro* p24 production was observed at 7 days after infection in peripheral blood mononuclear cells from white subjects with the 249D allele, although the difference was not statistically significant mainly due to the limited number of subjects with the mutant allele.[18] In addition, Old World monkey TRIM5α, including those of African green monkey, rhesus monkey, and cynomolgus monkey, also bears G at this position (Fig. 1). The HapMap project showed the 249D allele to be rare in whites (allele frequency: 0.053) but common in Japanese (allele frequency: 0.343) and African populations (allele frequency: 0.367). These findings prompted us to reevaluate the effects of this SNP on HIV-1 infection in Asians in which the frequency of G249D is higher than in whites.

To investigate the functional significance of G249D on the anti-HIV activity of TRIM5α, we constructed SeV containing C-terminal HA-tagged human TRIM5α (249G) (Fig. 1) by site-directed mutagenesis on MT4 TRIM5α, which bears D at position 249. As shown in Fig. 2A, the expression level of TRIM5α (249G) was comparable to that of TRIM5α (249D) in recombinant SeV-infected MT4 cells.

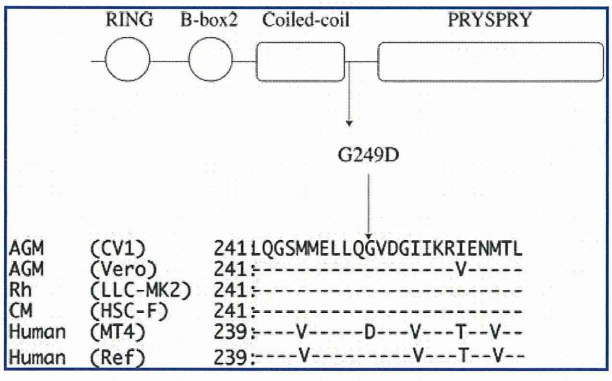

FIG. 1. Schematic presentation of TRIM5α structure. Circles and squares represent functional domains of TRIM5α. The position of the G249D polymorphism is shown by arrows. The amino acid sequences of African green monkey (AGM) TRIM5α from CV1[9] and Vero cells, rhesus monkey (Rh) TRIM5α from LLC-MK2,[10] cynomolgus monkey (CM) TRIM5α from HSC-F,[9] human TRIM5α from MT4 cells,[9] and the reference sequence (NM_033034) were aligned. Dashes denote an identical amino acid to AGM TRIM5α from CV1.

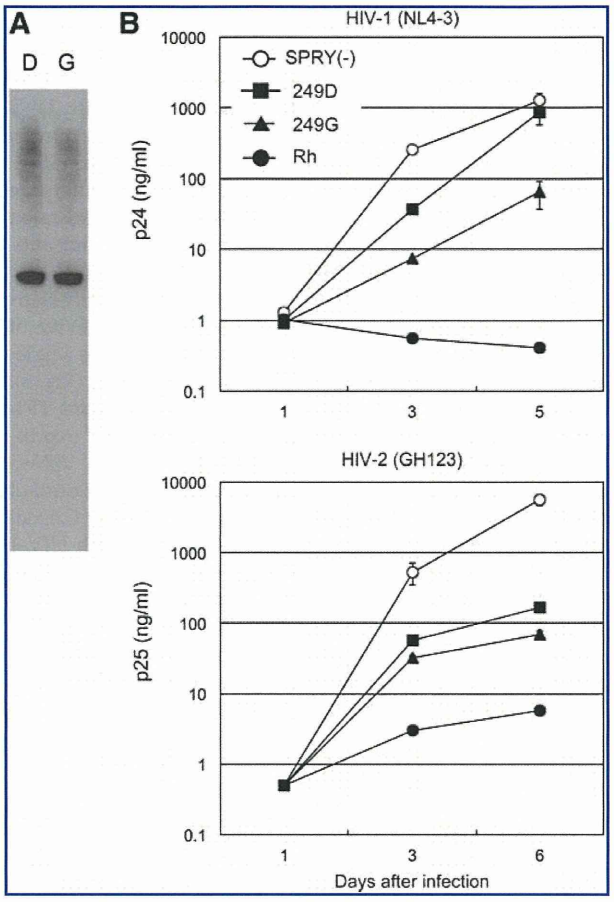

FIG. 2. **(A)** Lysates of MT4 cells infected with recombinant Sendai virus (SeV) expressing hemagglutinin (HA)-tagged human TRIM5α with 249D (lane D) and with 249G (lane G) were visualized by western blotting with an antibody against HA. Representative results of three independent experiments are shown. **(B)** MT4 cells were infected with SeV expressing TRIM5α lacking the PRYSPRY domain [SPRY(−); white circles], MT4-derived human TRIM5α (249D; black squares), human TRIM5α (249G; black triangles), or rhesus monkey TRIM5α (Rh; black circles). Nine hours after SeV infection, cells were inoculated with HIV-1 strain NL4-3 or HIV-2 strain GH123, and culture supernatants were periodically assayed for levels of p24 or p25, respectively. Data points are means for triplicate samples with SD. Three and six days after infection, statistically significant differences ($p < 0.05$) of HIV-1 and HIV-2 growth were observed between human TRIM5α (249D) and human TRIM5α (249G) by unpaired *t* test. Representative data of at least three independent experiments are shown.

These TRIM5α constructs were tested for their ability to restrict the X4-tropic HIV-1 strain NL4-3 and HIV-2 strain GH123. MT4 cells infected with recombinant SeV expressing each of the TRIM5α constructs were superinfected with HIV-1 NL4-3 or HIV-2 GH123. We used SeV expressing cynomolgus monkey TRIM5α lacking the PRYSPRY domain as a negative control for functional TRIM5α, as overexpression of TRIM5α lacking the PRYSPRY domain was shown to exert a dominant negative effect on endogenous human TRIM5α.[30] As shown in Fig. 2B, MT4-derived human TRIM5α (249D) showed only

weak anti-HIV-1 activity, as we demonstrated previously.[21] On the other hand, human TRIM5α (249G) showed stronger restriction activity to HIV-1 NL4-3 than human TRIM5α (249D). In the case of HIV-2, both human TRIM5α with 249G and 249D exhibited apparent anti-HIV-2 activity. The human TRIM5α (249G) showed stronger restriction activity to HIV-2 GH123 than human TRIM5α (249D), although the difference was very small (Fig. 2B, lower panel). These results indicated that the G249D variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α.

TRIM5α is known to restrict viral infection at the early steps of HIV replication. To evaluate the anti-HIV-1 activity of human TRIM5α at the early stages, we performed the single-round infection assay using a GFP expression vector (Fig. 3). The hamster cell line TK-ts13, which lacks endogenous TRIM5α expression, was infected with recombinant SeV expressing human TRIM5α. We superinfected cells with VSV-G pseudotyped lentivector expressing GFP under the control of the CMV promoter. We used HIV-1 vectors bearing CA derived from BH10 (Fig. 3A) and NL4-3 (Fig. 3B). Both HIV-1 GFP vectors were suppressed to a greater degree by human TRIM5α (249G) than by MT4-derived human TRIM5α (249D). A similar result was obtained when we used the HIV-2 GFP vector (Fig. 3C). Taken together, these observations indicated that the G249D polymorphism affected the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α.

Associations of TRIM5α G249D polymorphism with susceptibility to HIV-1 infection

We sequenced TRIM5α exon 5 and found G249D in the populations tested. The associations of G249D polymorphism with susceptibility to HIV-1 infection are summarized in Table 1. The frequency of 249D was significantly higher in the HIV-1-infected Indian subjects than in the ethnicity-matched controls [odds ratio (OR) = 1.52, $p = 0.026$]. A similar tendency was also observed in the Japanese population, but did not reach statistical significance (OR = 1.19, $p = 0.302$).

Previously, we sequenced TRIM5α exons 2 of the same subjects as above and reported the association of H43Y with susceptibility to HIV-1 infection.[21] The levels of LD indicated that G249D in exon 5 and H43Y in exon 2 were not in tight linkage disequilibrium in either Japanese ($r^2 = 0.18$, $n = 188$) or Indian ($r^2 = 0.02$, $n = 96$) populations.

Discussion

The G249D polymorphism in TRIM5α is common in Asian and African populations. It was initially speculated that there was no functional effect of this SNP, as it is located outside of any functional domains of human TRIM5α. Contrary to our expectation, however, we observed attenuation of anti-HIV-1 and anti-HIV-2 activity of the G-for-D substitution with both multiround replication and single-round infection assays. Furthermore, we investigated two ethnic populations, Japanese and Indian, for the G249D polymorphism and found the association of the TRIM5α 249D allele with enhanced susceptibility to HIV-1 infection.

Amino acid position 249 of human TRIM5α lies within the linker region for which no three-dimensional structural data have yet been reported. Therefore, we performed secondary structure prediction by the Chou–Fasman method[31] to examine the possible effect of this SNP on the protein structure.

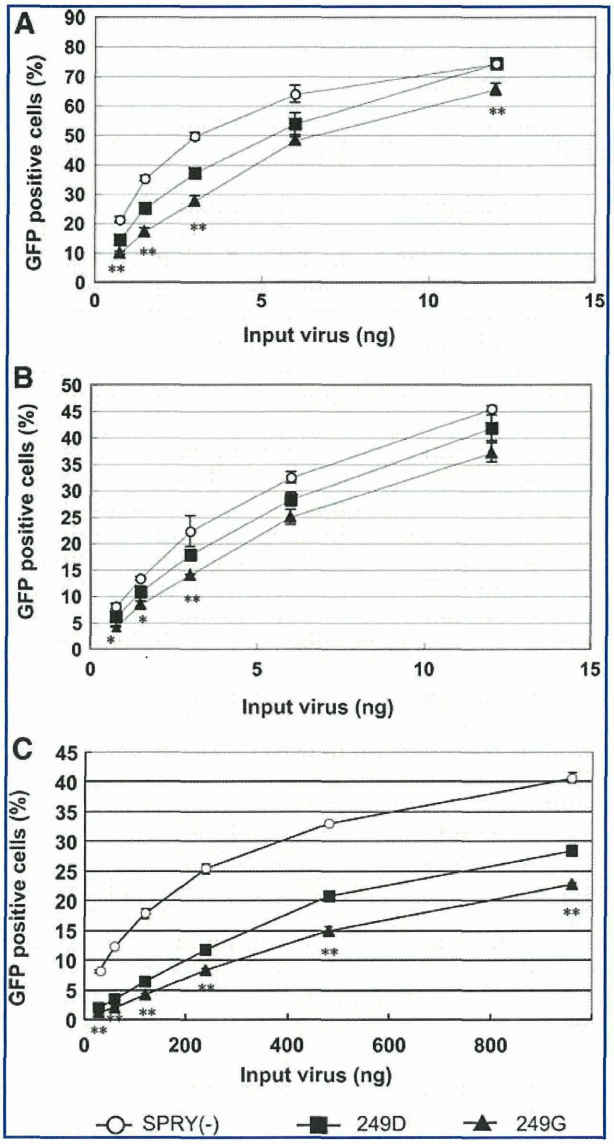

FIG. 3. TK-ts13 cells infected with SeVs expressing TRIM5α lacking the PRYSPRY domain [SPRY(−); white circles], MT4-derived human TRIM5α (249D; black squares), or human TRIM5α (249G; black triangles) were exposed to green fluorescence protein (GFP)-expressing HIV-1 vector based on BH10 **(A)** or NL4-3 **(B)**. **(C)** Cf2Th cells infected with SeVs were exposed to an HIV-2 vector based on ROD. GFP-positive cells were counted by a flow cytometer. Data points are means for triplicate samples with SD. *,** The statistically significant differences, $p < 0.05$ and $p < 0.001$, respectively, in unpaired t test between human TRIM5α (249D) and human TRIM5α (249G). Representative results of two independent experiments are shown.

The G-to-D substitution increased the probability of α-helix formation and resulted in the extension of the α-helix from the coiled-coil region into the linker 2 region. Similar results were obtained by the PREDETOR in http://mobyle.pasteur.fr (data not shown). This suggested that TRIM5α with 249G would be more flexible than TRIM5α with 249D.

TABLE 1. ASSOCIATION OF rs10038628 (G249D) WITH SUSCEPTIBILITY TO HIV-1 INFECTION IN JAPANESE AND INDIAN POPULATIONS

	Japanese				*Indian*			
	HIV-1-infected (n=93)	*Control* (n=279)	*Odds ratio (95% CI)*	p-*value*	*HIV-1-infected* (n=227)	*Control* (n=280)	*Odds ratio (95% CI)*	p-*value*
rs10038628								
GG	28 (30%)	98 (35%)	0.80 (0.48–1.32)	0.376	161 (71%)	226 (81%)	0.58 (0.39–0.88)	0.010
DG	47 (51%)	137 (49%)			63 (28%)	49 (17%)		
DD	18 (19%)	44 (16%)	1.28 (0.70–2.35)	0.422	3 (1%)	5 (2%)	0.74 (0.17–3.12)	0.736[a]
Allele D	83 (45%)	225 (40%)	1.19 (0.85–1.67)	0.302	69 (15%)	59 (11%)	1.52 (1.05–2.21)	0.026

[a]Fisher's exact test.

Human TRIM5α was obviously not effective in protecting against HIV-1 infection compared with the strong Old World monkey TRIM5α, as only humans are susceptible and Old World monkeys are resistant to HIV-1 infection. With experimental overexpression of human TRIM5α, the anti-HIV-1 activity of human TRIM5α was variable among previous reports.[1,5,9,14,16,20,21] Our previous data showed the weakest anti-HIV-1 activity of human TRIM5α,[9,20,21] even though we used the SeV system, which allowed high expression levels of inserted genes. As described in the present study, the 249D substitution would explain why our human TRIM5α derived from MT4 showed little potency against HIV-1. We examined the G249D SNP in commonly used human cell lines, CEM, HeLa, Jurkat, and 293T, and found that these were all homozygous for 249G, but MT4 was homozygous for 249D. This is not surprising because the allele frequency of 249D is high in Japan but quite rare in whites and MT4 cells were established from Japanese donor blood.[32] On the other hand, MT4 is highly susceptible to HIV-1 infection,[33] which is in good agreement with the present data.

Previously, Goldschmidt *et al.* failed to observe the attenuation of antiviral activity by the 249D mutation.[18] One possible reason for the discrepancy between their results and ours is the difference in expression system used. Goldschmidt *et al.* used HeLa cells stably transduced with TRIM5α with various mutations.[18] Transduced cell lines sometimes develop unexpected phenotypic changes during the cloning procedure. In contrast, we used the SeV system, and the conditions of cells infected with different recombinant viruses were always comparable, especially among those expressing full-length TRIM5α. It should be noted that Goldschmidt *et al.* also reported a tendency toward higher *in vitro* p24 production 7 days after infection in peripheral blood mononuclear cells from individuals with the 249D allele, which is consistent with our present results.[18]

We clearly showed that the 249D allele was associated with increased susceptibility to HIV-1 infection in the Indian population. However, although a similar tendency was observed in the Japanese population, the association was not significant. The precise reason why the effect of G249D was unclear in the Japanese population is not yet clear. It should be noted that our Japanese patients were infected through contaminated blood products in the early 1980s. On the other hand, the Indian patients were infected through heterosexual contact after the HIV-1 pandemic in Asia after 1990. It is possible that the difference in route of HIV-1 transmission may be responsible for this difference between Japanese and Indian patients. Further studies in well-characterized cohorts are necessary to confirm our findings regarding HIV-1 transmission and the possible effects of this SNP on AIDS progression.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, and Sodroski J: The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 2004;427:848–853.
2. Stremlau M, Perron M, Lee M, *et al.*: Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. Proc Natl Acad Sci USA 2006;103:5514–5519.
3. Perron MJ, Stremlau M, Song B, Ulm W, Mulligan RC, and Sodroski J: TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. Proc Natl Acad Sci USA 2004;101:11827–11832.
4. Yap MW, Nisole S, Lynch C, and Stoye JP: Trim5alpha protein restricts both HIV-1 and murine leukemia virus. Proc Natl Acad Sci USA 2004;101:10786–10791.
5. Perez-Caballero D, Hatziioannou T, Yang A, Cowan S, and Bieniasz PD: Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. J Virol 2005;79:8969–8978.
6. Sawyer SL, Wu LI, Emerman M, and Malik HS: Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. Proc Natl Acad Sci USA 2005;102:2832–2837.
7. Stremlau M, Perron M, Welikala S, and Sodroski J: Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. J Virol 2005;79:3139–3145.
8. Yap MW, Nisole S, and Stoye JP: A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. Curr Biol 2005;15:73–78.

9. Nakayama EE, Miyoshi H, Nagai Y, and Shioda T: A specific region of 37 amino acid residues in the SPRY (B30.2) domain of African green monkey TRIM5alpha determines species-specific restriction of simian immunodeficiency virus SIVmac infection. J Virol 2005;79:8870–8877.
10. Kono K, Song H, Shingai Y, Shioda T, and Nakayama EE: Comparison of anti-viral activity of rhesus monkey and cynomolgus monkey TRIM5alphas against human immunodeficiency virus type 2 infection. Virology 2008;373: 447–456.
11. Perron MJ, Stremlau M, and Sodroski J: Two surface-exposed elements of the B30.2/SPRY domain as potency determinants of N-tropic murine leukemia virus restriction by human TRIM5alpha. J Virol 2006;80:5631–5636.
12. O'Brien SJ and Nelson GW: Human genes that limit AIDS. Nat Genet 2004;36:565–574.
13. Shioda T and Nakayama EE: Human genetic polymorphisms affecting HIV-1 diseases. Int J Hematol 2006;84:12–17.
14. Javanbakht H, An P, Gold B, *et al.:* Effects of human TRIM5alpha polymorphisms on antiretroviral function and susceptibility to human immunodeficiency virus infection. Virology 2006;354:15–27.
15. Price H, Lacap P, Tuff J, *et al.:* A TRIM5alpha exon 2 polymorphism is associated with protection from HIV-1 infection in the Pumwani sex worker cohort. AIDS 2010;24: 1813–1821.
16. Sawyer SL, Wu LI, Akey JM, Emerman M, and Malik HS: High-frequency persistence of an impaired allele of the retroviral defense gene TRIM5alpha in humans. Curr Biol 2006;16:95–100.
17. Speelmon EC, Livingston-Rosanoff D, Li SS, *et al.:* Genetic association of the antiviral restriction factor TRIM5alpha with human immunodeficiency virus type 1 infection. J Virol 2006;80:2463–2471.
18. Goldschmidt V, Bleiber G, May M, Martinez R, Ortiz M, and Telenti A: Role of common human TRIM5alpha variants in HIV-1 disease progression. Retrovirology 2006;3:54.
19. van Manen D, Rits MA, Beugeling C, van Dort K, Schuitemaker H, and Kootstra NA: The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection. PLoS Pathog 2008;4:e18.
20. Nakayama EE, Carpentier W, Costagliola D, *et al.:* Wild type and H43Y variant of human TRIM5alpha show similar anti-human immunodeficiency virus type 1 activity both in vivo and in vitro. Immunogenetics 2007;59:511–515.
21. Nakajima T, Nakayama EE, Kaur G, *et al.:* Impact of novel TRIM5alpha variants, Gly110Arg and G176del, on the anti-HIV-1 activity and the susceptibility to HIV-1 infection. AIDS 2009;23:2091–2100.
22. Song H, Nakayama EE, Yokoyama M, Sato H, Levy JA, and Shioda T: A single amino acid of the human immunodeficiency virus type 2 capsid affects its replication in the presence of cynomolgus monkey and human TRIM5alphas. J Virol 2007;81:7280–7285.
23. Nakayama EE, Tanaka Y, Nagai Y, Iwamoto A, and Shioda T: A CCR2-V64I polymorphism affects stability of CCR2A isoform. AIDS 2004;18:729–738.
24. Miyoshi H, Takahashi M, Gage FH, and Verma IM: Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. Proc Natl Acad Sci USA 1997;94: 10319–10323.
25. Miyoshi H, Blomer U, Takahashi M, Gage FH, and Verma IM: Development of a self-inactivating lentivirus vector. J Virol 1998;72:8150–8157.
26. Kuroishi A, Bozek K, Shioda T, and Nakayama EE: A single amino acid substitution of the human immunodeficiency virus type 1 capsid protein affects viral sensitivity to TRIM5 alpha. Retrovirology 2010;7:58.
27. Miyamoto T, Nakayama EE, Yokoyama M, *et al.:* The carboxyl-terminus of human immunodeficiency virus type 2 circulating recombinant form 01_AB capsid protein affects sensitivity to human TRIM5alpha. PLoS One 2012;7:e47757.
28. Munkanta M, Terunuma H, Takahashi M, *et al.:* HLA-B polymorphism in Japanese HIV-1-infected long-term surviving hemophiliacs. Viral Immunol 2005;18:500–505.
29. Nakajima T, Ohtani H, Naruse T, *et al.:* Copy number variations of CCL3L1 and long-term prognosis of HIV-1 infection in asymptomatic HIV-infected Japanese with hemophilia. Immunogenetics 2007;59:793–798.
30. Maegawa H, Nakayama EE, Kuroishi A, and Shioda T: Silencing of tripartite motif protein (TRIM) 5alpha mediated anti-HIV-1 activity by truncated mutant of TRIM5alpha. J Virol Methods 2008;151:249–256.
31. Chou PY and Fasman GD: Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol Relat Areas Mol Biol 1978;47:45–48.
32. Akagi T, Ohtsuki Y, Shiraishi Y, and Miyoshi I: Transformation of human fetal thymus and spleen lymphocytes by human T-cell leukemia virus type I. Acta Med Okayama 1985;39:155–159.
33. Harada S, Koyanagi Y, and Yamamoto N: Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 1985;229:563–566.

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A novel link of *HLA* locus to the regulation of immunity and infection: *NFKBIL1* regulates alternative splicing of human immune-related genes and influenza virus *M* gene

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ABSTRACT

HLA locus contains immune-related genes and genetically regulates immune responses against both foreign- and self-antigens in humans. Inhibitor of κB-like protein (IκBL), encoded by *HLA*-linked *NFKBIL1*, is a protein of unknown function, while genetic variations in *NFKBIL1* are known to associate with the susceptibility to inflammatory and/or autoimmune diseases. In this study, we found that IκBL suppressed exon exclusion in alternative splicing of human immune-related genes such as *CD45*. Yeast-two-hybrid screening and immunoprecipitation assay revealed molecular association of IκBL with CLK1, a serine/threonine and tyrosine kinase, which plays a role in the alternative splicing. Unexpectedly, we found that the regulation of alternative splicing in *CD45* by IκBL was independent from the kinase activity of CLK1. On the other hand, it was demonstrated that an SR protein, ASF/SF2, bound both IκBL and CLK1 at the RNA-recognition motifs of ASF/SF2, implying a competition of IκBL and CLK1 on SR protein. In addition, IκBL was found to regulate the CLK1-dependent synthesis of *M2* RNA, a splice variant of influenza A virus *M* gene. These observations suggest a functional involvement of IκBL in the regulation of alternative splicing in both human and viral genes, which is a novel link of *HLA* locus to the regulation of immunity and infection in humans.

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1. Introduction

Human NF-κB inhibitor-like protein 1 gene (*NFKBIL1*) is located in the *HLA* class III region on the short arm of chromosome 6 and encodes a protein, inhibitor of κB-like protein (IκBL), which shows a limited homology to inhibitor of κB (IκB). A number of studies have demonstrated the association between genetic variations in *NFKBIL1* and susceptibility to inflammatory and/or autoimmune diseases, such as multiple sclerosis [1], rheumatoid arthritis (RA) [2], type I diabetes mellitus [3], Takayasu's arteritis [4], and chronic thromboembolic pulmonary hypertension [5]. It has been reported that the sequence variations in the promoter region of *NFKBIL1*, which showed the lowest and the highest promoter activity, would confer the susceptibility to RA and Takayasu's arteritis, respectively [4], implying that the altered expression of IκBL might contribute to the pathogenesis of immune-related diseases.

Alternative splicing is a crucial mechanism in the post-transcriptional control of gene expression in eukaryotes, in which target exons in pre-mRNA could be either excluded or included depending on the specific *cis*-regulatory elements and recognition by the splicing-related factors [6,7]. Pre-mRNAs of human immune-related genes are known to undergo extensive alternative splicing [8]. For example, resting T cells express larger mRNA isoforms of *CD45*, while target exons of *CD45* were selectively excluded to form shorter mRNA isoforms in activated T cells. Expression of each isoform generated by the alternative splicing is strictly regulated, whereas the abnormal alternative splicing events, which lead to altered expression of target mRNA isoforms, have been reported to associate with autoimmune diseases [9,10].

IκBL possesses nuclear localization sequences (NLS) at its N-terminus and localized in nuclear speckles [11,12], which are sub-nuclear structures enriched with pre-mRNA splicing factors [13]. In addition, IκBL was reported to associate with RNA [12]. These lines of evidence suggest that IκBL might play a role in the RNA splicing. In addition, it has been reported that CDC-like kinase 1 (CLK1),

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a serine/threonine and tyrosine kinase, localizes in nuclear speckles and nucleoplasm, and CLK1 regulates alternative splicing through phosphorylation of serine/arginine rich (SR) proteins [14–16]. SR proteins play an important role in both constitutive splicing and alternative splicing of pre-mRNA [6,7]. Functional domain of SR proteins contains one or two RNA-recognition motifs (RRMs), where pre-mRNAs were bound to be processed.

We report here that IκBL physically interacts with CLK1 and SR protein, and functions as a novel regulator in the alternative splicing of both human and viral genes.

2. Materials and methods

2.1. Calculation of Ka/Ks value for amino acid substitution

Ka/Ks value was used to estimate the potential functional domain of IκBL. The Ka and the Ks values are calculated by DnaSP v3.0 [17], by comparing the human and murine *NFKBIL1* sequences. Ks is the number of synonymous substitutions per synonymous site, whereas Ka is the number of nonsynonymous substitutions per nonsynonymous site.

2.2. Plasmids

An exon trapping vector, pSPL3 (Invitrogen, Carlsbad, CA, USA), was used to construct mini-genes analyzed for the alternative splicing. A *CD45* mini-gene construct covered exons 3–7 and their intron–exon boundary segments from human *CD45*, while *CD72* and *CTLA4* mini-gene constructs encompassed exons 7–8, and exons 2–4, respectively, with their intron–exon boundary segments from human the genes. We cloned human cDNAs encoding IκBL, CLK1, hnRNPLL, hnRNPL, FOX1 and ASF/SF2 into mammalian expression vectors, pCI-neo (Promega, Madison, WI, USA) and pEGFP (Clontech, Mountain View, CA, USA). Deletion mutants of IκBL (IκBL-ΔN, -ΔA, -ΔCv and -ΔCc), CLK1 (CLK1-ΔN, -Δkinase) and ASF/SF2 (ASF/SF2-ΔRRM1β1, -ΔRRM2β1, -ΔRRM1&2β1 and -ΔRS) were generated by the standard PCR-based method. All constructs were sequenced to ensure that undesired mutations were not introduced during the cloning procedure. The constructs used in the plasmid-based rescue system for the influenza A virus, including pPOLI-M-RT, pcDNA-NP, pcDNA-PB1, pcDNA-PB2 and pcDNA-PA, were kindly provided by Dr. George G. Brownlee and Dr. Ervin Fodor. Plasmid DNAs for transfection were prepared using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany).

2.3. Cell culture and transfection

COS7, HeLa and HEK293T cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% de-complemented fetal calf serum (FCS) (Nichirei Biosciences, Tokyo, Japan) and Penicillin–Streptomycin–Glutamine (PSG) (Invitrogen). JSL1 cells were kindly provided by Dr. Kristen W. Lynch and maintained in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 5% FCS plus PSG. Transfection was done using COSFectin (Bio-Rad, Hercules, CA, USA) or Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. Hygromycin (Invitrogen) was used for selection of a stably transfected JSL1 line, JSL-IκBL.

2.4. RNA interference

Knockdown of endogenous *NFKBIL1* and *CLK1* was done by using pre-designed siRNAs (siRNA ID for *NFKBIL1*: s9517 and s194653; siRNA ID for *CLK1*: s3162 and s3163) (Ambion, Austin, TX, USA). A non-targeting siRNA was used as a negative control.

2.5. Immunofluorescence staining

Fixed and permeabilized HeLa cells were incubated with anti-SC35 (BD Biosciences Pharmingen, San Diego, CA, USA) and/or anti-CLK1 (Abcam, Cambridge, MA, USA) antibodies, followed by incubation with fluorescence-conjugated second antibodies. Images were analyzed with an LSM510 laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.6. RNA isolation, RT-PCR and real-time RT-PCR

Total cellular RNAs from human tissues were purchased from Agilent Technologies. Total RNAs from cells were purified by using RNeasy Mini kit (Qiagen) and cDNAs were synthesized by the reverse transcription (RT) reaction from 1 μg of RNA using PrimeScript RT reagent Kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. To evaluate the amount of splicing variants, cDNA was applied to PCR and the PCR products were separated by electrophoresis on agarose gels, visualized by ethidium bromide staining, and quantified by using ImageJ Version 1.36. The endogenous expression of mRNA was quantified by real-time RT-PCR using iCycler iQ Real-Time PCR Detection System (Bio-Rad).

2.7. Yeast-two-hybrid (Y2H) screening

All procedures for Y2H were performed according to the manufacturer's instructions for the Matchmaker GAL4 Two-Hybrid System 3 (Clontech).

2.8. Immunoprecipitation (IP) and immunoblotting

IP products were prepared by precipitation of antigen–antibody complex using Protein G Sepharose beads (GE Healthcare, Uppsala, Sweden). For immunoblotting, samples were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Invitrogen). After the incubation with antibodies, signals were visualized by Image Reader LAS-3000 (FUJIFILM, Tokyo, Japan).

2.9. Flow cytometry analysis

JSL1 and JSL1-IκBL cells with or without activation by 12-myristate 13-acetate (PMA) (Calbiochem, San Diego, CA, USA) were incubated with specific antibodies to CD45RA (eBioscience, San Diego, CA, USA) or CD45RO (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Flow cytometry analysis was performed on FACS-Calibur (BD Biosciences, San Jose, CA, USA) according to the standard protocol.

2.10. Statistical analysis

Statistical comparisons were performed using Student's *t*-tests or one-way ANOVA followed by a post-hoc Bonferroni's or Dunnett's multiple comparison tests. The results were considered statistically significant when the p value was less than 0.05.

Additional information to *2.4.*, *2.5.*, *2.7.*, *2.8.* and antibodies used in this study can be found in Supplementary information.

3. Results

3.1. Domain structure of IκBL for localization to nuclear speckles

Based on the domain structure and Ka/Ks value, IκBL was divided into four segments; N-terminal segment (N) (amino acids 1–66) containing a putative NLS, ankyrin repeat domain segment (A) (amino acids 67–137), central variable segment (Cv)

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(amino acids 138–297), and C-terminal conserved segment (Cc) containing a leucine zipper motif (amino acids 298–381) (Fig. 1A and B). Cellular localization of IκBL was investigated in HeLa cells transfected with EGFP-tagged IκBL. As shown in Fig. 1C, IκBL-linked EGFP signal was co-localized with SC35, a member of SR protein family, in the nuclear speckles. On the other hand, HeLa cells expressing EGFP-IκBL-ΔN showed diffuse cytoplasmic EGFP signals, demonstrating that the segment N was essential for the nuclear localization. Segments A and Cv were indispensable for the nuclear localization of IκBL, because their deletions impaired the localization to the nuclear speckles. In contrast, deletion of the segment Cc had no effect on the subnuclear localization of IκBL.

3.2. IκBL inhibits exon exclusion in alternative splicing of immune-related genes

Localization of IκBL in the nuclear speckles, along with the evidence that genetic variations of IκBL were associated with the susceptibility to inflammatory and/or autoimmune diseases, leads to a hypothesis that IκBL might play a pivotal role in the alternative splicing of immune-related genes. Because human *CD45* gene is known to undergo alternative splicing of exons from 3 to 7, we generated a mini-gene construct for human *CD45* covering exons 3–7. The mini-gene construct was transfected into a monkey cell line COS7 with inducers of alternative splicing, hnRNPLL or hnRNPL [18–21]. In the hnRNPLL-induced *CD45* alternative splicing, IκBL decreased the generation of exons 3-7 isoform and oppositely increased the exons 3-4-5-6-7 isoform (Fig. 2A). Similar effects of IκBL on the *CD45* alternative splicing were also observed in human cell lines, HeLa and HEK293T (data not shown). In addition, it was observed that IκBL also suppressed the hnRNPL-induced alternative splicing of *CD45* (Supplementary Fig. S1).

We next examined whether the silencing of endogenous *NFKBIL1* would affect the hnRNPLL-induced *CD45* alternative splicing. *CD45* mini-gene and hnRNPLL were transfected into HeLa, in which the endogenous *NFKBIL1* was interfered by human *NFKBIL1*-specific siRNA. It was found that the knock-down of *NFKBIL1* increased the exons 3-7 isoform and concomitantly decreased the exons 3-5-7 isoform (Fig. 2B), indicating that IκBL facilitated the exon inclusion in alternative splicing of *CD45*.

To examine the effect of IκBL on other human immune-related genes, we created mini-genes of *CD72* and *CTLA4*. A *CD72* mini-gene covered exons 7 and 8, whereas a *CTLA4* mini-gene encompassed exons 2–4. The hnRNPLL-induced *CD72* alternative splicing was counteracted by the expression of IκBL (Supplementary Fig. S2A). On the other hand, hnRNPLL-induced *CD72* alternative splicing was accelerated in cells where the endogenous *NFKBIL1* was silenced (Supplementary Fig. S2B). In addition, we found a suppression of FOX1-induced *CTLA4* alternative splicing by IκBL (Supplementary Fig. S3). Furthermore, we studied which domain of IκBL was involved in the regulation of alternative splicing. It was revealed that IκBL-ΔN, -ΔA and -ΔCv failed to suppress the hnRNPLL-induced *CD45* alternative splicing, whereas -ΔCc could suppress it similar to the intact (-FL) IκBL (Fig. 2C).

3.3. Identification of molecules interacting with IκBL by Y2H screening

Expression of *NFKBIL1* in human tissues was examined by real-time RT-PCR. It was found that *NFKBIL1* was ubiquitously expressed with the prominent expression in spleen (Supplementary Fig. S4A). Next, a Y2H screening of human spleen cDNA library was performed to identify interacting molecules

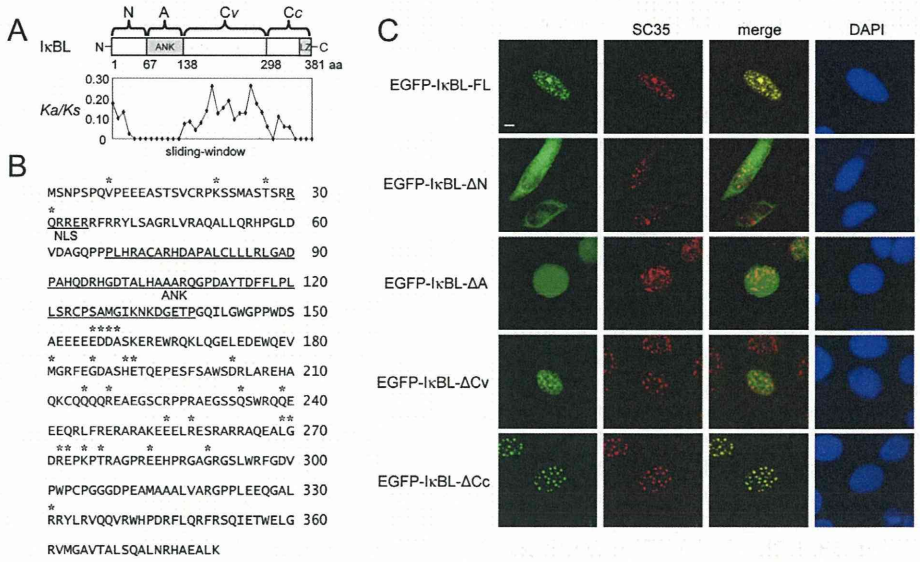

Fig. 1. Structure and cellular localization of IκBL. (A) Ka/Ks value based on the sliding window plot analysis for the *NFKBIL1* gene. Ks is the number of synonymous substitutions per synonymous site, whereas Ka is the number of nonsynonymous substitutions per nonsynonymous site, by comparing the human and murine *NFKBIL1* sequences. The Ka and the Ks values are calculated by DnaSP (v3.0). According to Ka/Ks value, IκBL was divided into four segments; N-terminal segment (N) (amino acids 1–66) containing a putative NLS, ankyrin repeats domain segment (A) (amino acids 67–137), central variable segment (Cv) (amino acids 138–297) and C-terminal conserved segment (Cc) with leucine zipper motif (amino acids 298–381). Amino acids are numbered starting from the first in-frame methionine codon. (B) Amino acid sequences of human IκBL. NLS and ankyrin repeats domain (ANK) are underlined. Asterisks indicate the positions of amino acids that are different from the amino acid sequences of murine IκBL. (C) HeLa cells were transfected with EGFP-IκBL-FL, -ΔN, -ΔA, -ΔCv or -ΔCc (EGFP signal, green) and immunofluorescence staining was performed by using anti-SC35 antibody (Alexa Fluor 568-labeled, red). IκBL-FL co-localized with SC35 in nuclear speckles. IκBL-ΔN localized in the cytosol. Both IκBL-ΔA and -ΔCv were found in the nuclei, but the localization to nuclear speckles was impaired. IκBL-ΔCc could localize to nuclear speckles, similar as IκBL-FL. A bar represents 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)