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SHORT COMMUNICATION

SLP65 deficiency results in perpetual V(D)J recombinase activity in pre-B-lymphoblastic leukemia and B-cell lymphoma cells

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Perpetual V(D)J recombinase activity involving multiple DNA double-strand break events in B-cell lineage leukemia and lymphoma cells may introduce secondary genetic aberrations leading towards malignant progression. Here, we investigated defective negative feedback signaling through the (pre-) B-cell receptor as a possible reason for deregulated V(D)J recombinase activity in B-cell malignancy. On studying 28 cases of pre-B-lymphoblastic leukemia and 27 B-cell lymphomas, expression of the (pre-) B-cell receptor-related linker molecule SLP65 (SH2 domain-containing lymphocyte protein of 65 kDa) was found to be defective in seven and five cases, respectively. SLP65 deficiency correlates with RAG1/2 expression and unremitting V_H gene rearrangement activity. Reconstitution of SLP65 expression in SLP65-deficient leukemia and lymphoma cells results in downregulation of RAG1/2 expression and prevents both *de novo* V_H–DJ_H rearrangements and secondary V_H replacement. We conclude that iterative V_H gene rearrangement represents a frequent feature in B-lymphoid malignancy, which can be attributed to SLP65 deficiency in many cases.
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Perpetual V(D)J recombinase activity continuously generates DNA double-strand breaks and may give rise to secondary transforming events during the malignant progression of early leukemia and lymphoma cells (Khanna and Jackson, 2001). In B-cell precursors, V(D)J recombination is regulated through a negative feedback signal: upon successful rearrangement, a μ-heavy chain encoded by a productively rearranged V_H region gene signals termination of recombination activity at the *IGHV* locus (Grawunder *et al*., 1995). How this negative feedback signal is deranged in leukemia and lymphoma cells is not yet resolved.

Recent work demonstrated that deficiency of SLP65 (SH2 domain-containing lymphocyte protein of 65 kDa) is a frequent feature in acute lymphoblastic leukemia cells (Jumaa *et al*., 2003; Klein *et al*., 2004). Although a recent report questioned these findings (Imai *et al*., 2004), this study shows that defective SLP65 expression is not only frequent in human pre-B-lymphoblastic leukemia but also occurs in a fraction of mature B-cell lymphoma cases. Identifying three leukemia and one lymphoma cell line lacking expression of functional SLP65, we studied the contribution of SLP65 to the control of the V(D)J recombinase activity in B-cell lineage leukemia and lymphoma cells.

Perpetual V(D)J recombinase activity in B-cell lineage leukemia and lymphoma cells

In order to investigate ongoing V(D)J recombinase activity in B-cell precursor leukemia and B-cell lymphoma cells, we first analysed the configuration of immunoglobulin (Ig) gene loci in leukemia and lymphoma cell lines. Among 22 clonal pre-B-lymphoblastic leukemia and B-cell lymphoma cell lines, five of 12 pre-B-lymphoblastic leukemia and two of 10 B-cell lymphoma cell lines express RAG1 and RAG2, and carry more than two productively rearranged Ig heavy chain V region genes, indicating that negative feedback signaling of the (pre-) B-cell receptor to V(D)J recombinase activity was impaired in these cells (Table 1). In (pre-) B-lymphoblastic cell lines harboring only one productively rearranged *IGHV* allele, expression of RAG1 and RAG2 does not necessarily indicate defective negative feedback signaling of the (pre-) B-cell receptor and may also reflect active rearrangement of *IGKV* and *IGLV* light chain genes. In addition, ongoing V(D)J recombinase activity represents a typical feature of pre-B-lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement as previously shown by us and others (Height *et al*., 1996; Klein *et al*., 2004), suggesting that BCR-ABL1 kinase activity interferes with negative feedback signaling of the pre-B-cell receptor (Klein *et al*., 2004).

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Table 1 (A) Pre-B-cell receptor configuration, V(D)J recombinase activity and SLP65 expression in pre-B-acute lymphoblastic leukemia cells

Case	*Entity*	*Chromosomal rearrangement*	*IGHV*	*CC*	*Recombinase activity*		*SLP65 WT*	*SLP65 mutations*	*SLP65 splice variants*
					RAG expression	*Active V_H rearrangement*			
BEL1	Pre-B ALL	*MLL-AF4*	V1-2 D3-22 J6	+	RAG1, RAG2	*De novo* V_H–DJ_H Secondary V_H replacement	No	N27S	ΔPRD (exons 5–8)
			V2-5 D2-2 J6	+					
			V3-13 D2-2 J6	+					
			V4-31 D2-2 J6	–					
			V7-4 D2-2 J6	–					
			V3-7 D3-22 J6	–					
			V3-13 D3-22 J6	–					
			V3-23 D3-22 J6	+					
			V3-30 D3-22 J6	–					
			V6-1 D3-22 J6	–					
			Germ line						
RS4;11	Pre-B ALL	*MLL-AF4*	V3-20 D2-8 J5	–	RAG1	None	Yes	ND	ND
			V6-1 D1-7 J4	–					
SEM	Pro-B ALL	*MLL-AF4*	D3-10 J5		None	None	Yes	None	None
			Germ line						
REH	Pre-B ALL	*TEL-AML1*	V3-15 D3-10 J6	–	RAG1	None	Yes	ND	ND
			Germ line						
BV173	Pre-B ALL	*BCR-ABL1*	V3-48 D2-2 J3	–	RAG1, RAG2	*De novo* V_H–DJ_H Secondary V_H replacement	No	L39P	ΔPRD, SH2 (exons 6–17)
			V3-38 D2-2 J3	–				E82K	ΔPRD, SH2 (exons 5–17)
			V3-21 D2-15 J3	–				S436F	INS introns 3–4
			D2-15 J3						
SUP-B15	Pre-B ALL	*BCR-ABL1*	V3-53 D2-8 J6-2	+	RAG1, RAG2	*De novo* V_H–DJ_H Secondary V_H replacement	Yes	G30S	ΔPRD, SH2 (exons 8–17)
			V1-2 D2-2 J6-2	–					ΔPRD, SH2 (exons 6–16)
			V4-4 J6-2	–					INS introns 3–4
			V6-1 D5-5 J6-2	+					
			Germ line						
Nalm1	Pre-B ALL	*BCR-ABL1*	V1-8 J2	–	RAG1, RAG2	*De novo* V_H–DJ_H Secondary V_H replacement	Yes	ND	ΔPRD, SH2 (exons 8–17
			V1-8 J4	–					INS introns 3–4
			V2-5 D3-16 J4	–					
			V2-70 D3-16 J4	–					
			V3-9 J6	+					
			V4-31 D5-24 J4	+					
			V4-34 D3-16 J4	–					
			V4-59 D3-16 J4	–					
			V4-61 D3-16 J4	+					
			V5-51 D3-16 J4	+					
			V6-1 D3-16 J4	+					
Nalm6	Pre-B ALL	*TEL-PDGFRB*	V1-69 D3-10 J6	+	RAG1	None	Yes	ND	ND
Kasumi-2	Pre-B ALL	*E2A-PBX1*	V3-7 D3-10 J4	+	RAG1	None	Yes	ND	ND
MHH-CALL3	Pre-B ALL	*E2A-PBX1*	V3-15 D3-16 J5	+	RAG1	None	Yes	ND	ND
697	Pre-B ALL	*E2A-PBX1*	V2-26 D2-2 J4	+	RAG1	None	Yes	ND	ND
HPB-NULL	Pre-B ALL	Hyperdiploid	V3-9 D3-22 J6	–	RAG1, RAG2	*De novo* V_H–DJ_H Secondary V_H replacement	No	P165S	ΔPRD, SH2 (exons 5 and 6)
			V4-59 D2-8 J6	+				W232R	ΔPRD, SH2 (exons 4–6)
			V6-1 D5-5 J6	+				T314A	
			V6-1 D6-25 J6	–					
			Germ line						

Table 1 (continued) (B) B-cell receptor configuration, V(D)J-recombinase activity and SLP65 expression in B-cell lymphoma cells

Case	*Entity*	*Chromosomal rearrangement*	*IGHV*	*CC*	*Recombinase activity*		*SLP65 WT*	*SLP65 mutations*	*SLP65 splice variants*
					RAG expression	*Active V_H rearrangement*			
MEC1	B-CLL	Hyperdiploid	V4-59 D3-3 J4	+	None	None	Yes	Deletion at 10q23	ΔPRD, SH2 (exons 4–6)
			V4-59 D2-21 J4	+					ΔPRD, SH2 (exons 8 and 9
			V2-70 D3-22 J3	–					ΔPRD, SH2 (Exon 6)
			V2-70 D3-22 J4	+					
Granta-519	MCL	*CCND1-IGH*	V4-59 D5-5 J4	+	None	None	Yes	None	None
Jeko-1	MCL	*CCND1-IGH*	V2-70 D3-3 J4	+	RAG1, RAG2	None	Yes	ND	ND
HBL-2	MCL	*CCND1-IGH*	V3-11 D3-22 J1	+	None	None	Yes	None	None
JVM-2	MCL	*CCND1-IGH*	V3-9 D6-19 J4	+	None	None	Yes	ND	ND
SP49	MCL	*CCND1-IGH*	V4-34 D3-22 J2	+	None	None	Yes	None	None
NCEB-1	MCL	*CCND1-IGH*	V3-53 D2-21 J6	+	None	None	Yes	ND	ND
MHH-PREB	Burkitt's	*MYC-IGH*	V4-34 D2-15 J5	+	RAG1, RAG2	None	Yes	ND	ND
			V3-53 D3-3 J6	–					
MC-116	Burkitt's	*MYC-IGH*	V1-2 D1-26 J4	+	None	None	Yes	None	None
Karpas-422	DLBCL	*BCL2-IGH*	V1-3 D3-3 J6	+	RAG1,	*De novo* V_H–DJ_H	No	Deletion at 10q23	ΔPRD, SH2 (exons 3 and 7–16)
			V1-18 D1-26 J4	+	RAG2	Secondary V_H replacement		LOH: Δ28bp in	ΔPRD, SH2 (exons 3 and 4)
			V1-18 D2-2 J6	+				Exon 3 and introns 3–4	ΔPRD, SH2 (exons 3–5)
			V2-70 D3-22 J3	+					ΔPRD, SH2 (exons 3 and 8–9)
			V3-7 D5-12 J4	+					
			V3-9 D3-22 J6	–					
			V3-33 D6-13 J4	–					
			V6-1 D3-22 J4	+					
			V3-73 D2-15 J4	+					
			V4-39 D3-10 J6	+					
			V4-39 J6	+					
			D3-22 J6						
			Germ line						
Normal B cells	None	None	Polyclonal	+/–	None	None	Yes	None	None

Abbreviations ALL, acute lymphoblastic leukemia; CC, coding capacity; DLBCL, diffuse large B-cell lymphoma; IGH, immunoglobulin heavy chain; LOH, loss of heterozygosity; ND, not determined; PRD, proline-rich domain; SH2, SRC-homology domaín 2; SLP65, SH2 domain-containing lymphocyte protein of 65 kDa; WT, wild type. *De novo* V_H–DJ_H rearrangement: rearrangement of a pre-existing DJ_H joint to a V_H gene segment. Secondary V_H replacement: replacement of a previously rearranged V_H segment within a V_HDJ_H joint by rearrangement of an upstream V_H segment to a cryptic RSS within the 3′ part of the previously rearranged V_H. From MEC1 cells, amplification of RAG1 transcripts yielded a weak band in one experiment, which could not be reproduced in three repeat experiments.

SLP65 deficiency in B-cell precursor leukemia and B-cell lymphoma cells

In murine B cells, the (pre-) B-cell receptor-associated linker molecule SLP65 is required to downregulate V(D)J recombinase activity (Hayashi *et al*., 2003) and acts as a tumor suppressor in pre-B-lymphoblastic leukemia cells (Jumaa *et al*., 2003). Studying SLP65 expression in B-cell precursor leukemia and B-cell lymphoma by Western blot, we found that expression of SLP65 protein was defective in seven of 28 leukemia cases (four of 16 primary cases and three of 12 cell lines; Figure 1a) and five of 27 lymphomas (four of 17 primary cases and one of 10 cell lines; Figure 1a). Sequence analysis revealed that SLP65 transcripts frequently lost their coding capacity for full-length SLP65 protein owing to aberrant splicing with exon skipping and usage of cryptic splice sites and splice site slippage (Table 1). In one case of diffuse large B-cell lymphoma (DLBCL) (Karpas-422), aberrant splicing was the result of a genomic deletion of the 3′ splice site of exon 3 of the *SLP65* gene (Figure 1b). Owing to a 28 bp deletion of the 3′ part of exon 3 and the 5′ part of introns 3–4, full-length SLP65 can no longer be expressed from this allele. Of note, the second *SLP65* allele in these DLBCL cells was lost owing to a large deletion at 10q23 (R Siebert, unpublished). Chromosomal deletion and loss of heterozygosity by somatic mutation is consistent with a role of *SLP65* as a tumor suppressor gene in these DLBCL cells. Sequence analysis of the coding region of *SLP65* and intronic splice sites revealed a number of other somatic mutations leading to amino-acid changes or loss of the reading frame (Table 1). Somatic mutations of the *SLP65* gene were amplified from BEL1, BV173, SUP-B15 and HPB-NULL cells (Table 1A). Non-functional SLP65 mRNA splice variants were amplified from all cases of B-cell lineage leukemia and lymphoma lacking negative feedback signaling through the (pre-) B-cell receptor (ongoing RAG expression together with multiple V_H gene rearrangements; Table 1). These findings suggest that SLP65 is required to halt the recombination machinery upon successful VDJ rearrangement at the *IGHV* locus.

V_H replacement in SLP65-deficient leukemia and lymphoma cells

As previously shown by us and others (Zhang *et al*., 2003; Klein *et al*., 2004), perpetual V(D)J recombinase activity may involve *de novo* V_H to DJ_H rearrangements or secondary rearrangements by replacement of a previously rearranged V_H gene segment by a yet unrearranged upstream V_H gene segment. In this case, a previously rearranged V_H gene segment is cleaved at a cryptic recombination signal sequence (RSS) in its 3′ part with only 5–7 bp remaining as a relict of the initially rearranged V_H gene segment. Such footprints could indeed be detected in the *IGH* (immunoglobulin heavy chain) VDJ rearrangements of five pre-B-lymphoblastic leukemia cell lines (BEL1, BV173, SUP-B15, Nalm1 and HPB-NULL) and one B-cell lymphoma cell line (Karpas-422; Table 2). In two of these five cell lines, the leukemia cells exhibit expression of SLP65. Ongoing V(D)J recombinase activity in these two cases (SUP-B15 and Nalm1), despite expression of SLP65, reflects that these leukemia cells express the oncogenic BCR-ABL1 kinase, which interferes with negative feedback signaling of the pre-B-cell receptor (Klein *et al*., 2004).

To test if *de novo* rearrangement and V_H replacement are caused by SLP65 deficiency, we reconstituted SLP65 expression in SLP65-deficient pre-B-lymphoblastic leukemia cells (BEL1) and diffuse large B-cell lymphoma cells (Karpas-422) by nucleofection. After 2 days, SLP65-reconstituted cells were sorted and analysed for expression of RAG1 and RAG2 and the presence of short-lived DNA double-strand break intermediates at RSSs flanking V_H and J_H gene segments.

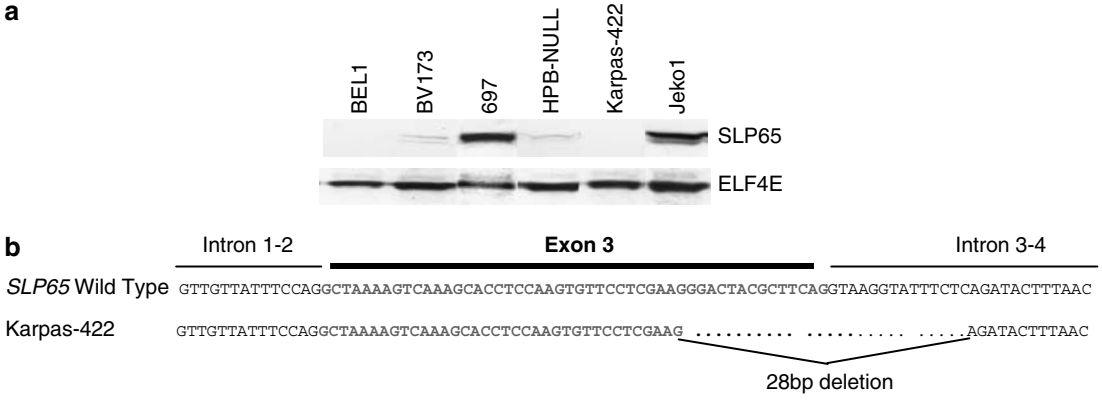

Figure 1 SLP65 deficiency in B-cell precursor leukemia and B-cell lymphoma cells. Western blot analysis shows defective SLP65 expression in BEL1, BV173, HPB-NULL and Karpas-422 cells. EIF4E was used as a loading control (**a**). Sequence analysis of one allele of *SLP65* in Karpas-422 diffuse large B-cell lymphoma cells reveals a 28 bp deletion of the 3′ part of exon 3 and the 5′ part of introns 3–4 (**b**). Amplification and sequencing of *SLP65* was performed as described previously (Feldhahn *et al*., 2005), using the primer pairs listed in Supplementary Table 1. Sequence data is available from EMBL/GenBank under accession number AM180347. The second *SLP65* allele is missing in a classical loss of heterozygosity situation owing to a large chromosomal deletion at 10q23. A detailed description of the cell lines used is given in Table 1 and in Supplementary information.

Table 2 Analysis of *de novo* V_H to DJ_H rearrangement and secondary V_H gene replacement in V_H region genes of pre-B-lymphoblastic leukemia and B-cell lymphoma cells

Case	*3′ of recipient*	*V_H–D_H junction*	*3′ of donor V_H*		*V_H–D_H junction*	*D_H–J_H junction*	
BEL1, allele 1	V_H4-31	CGTGTATTACTGTGCGAGA GAGGGGTTTGAGCGGG	V_H3-13	TGTGTATTACTGTGCAAGAGA	GGGTTTGAGCGGG	TATTGTAGTAGTACCAGCTGC	D_H2-2 J_H6
	V_H4-31	CGTGTATTACTGTGCGAGA GAGGGGTTTGAGCGGG	V_H2-5	CACATATTACTGTGCACAC	CCCCCCGGGGGGGGG TTTGAGCGGG	TATTGTAGTAGTACCAGCTGC	D_H2-2 J_H6
	V_H4-31	CGTGTATTACTGTGCGAGA GAGGGGTTTGAGCGGG	V_H7-4	CGTGTATTACTGTGCGAGA	GGGTTTGAGCGGG	TATTGTAGTAGTACCAGCTGC	D_H2-2 J_H6
BEL1, allele 2 (*de novo* V_H to DJ_H rearrangement)							
	—		V_H3-30	TGTGTATTACTGTGCGAGAGA	TA	ATTACTATGATAGTAGTGG	D_H3-22 J_H6
	—		V_H3-23	CGTATATTACTGTGCGA	TCCTCG	AGTGG	D_H3-22 J_H6
	—		V_H3-13	TGTGTATTACTGTGCAAGAGA		TACTATGATAGTAGTGG	D_H3-22 J_H6
	—		V_H3-7	TGTGTATTACTGTGCGAGAGA	C	GTATTACTATGATAG TAGTGG	D_H3-22 J_H6
	—		V_H1-2	CGTGTATTACTGTGCGA GAGA		TATTACTATGATAGTAGTGG	D_H3-22 J_H6
	—		V_H6-1	TGTGTATTACTGTGCAAGAGA	CCGTATAGCAGTGGCTG	GTATTACTATGATAG TAGTGG	D_H3-22 J_H6
BEL1, allele 3 (germ line)							
NALM1, allele 1	V_H1-45	CATGTATTACTGTGCAAGATA	V_H1-69	GTGTATTACTGTGCGAGAG	GTCAA	GATATTGTAGTGGTGG TAGCTGCT	D_H2-15 J_H 3
NALM1, allele 2 (*de novo* V_H to DJ_H rearrangement)							
	—		V_H2-5	ACATATTACTGTGCACACA GATCG		ACACAGATCGGGGGG TACTTTG	D_H3-16 J_H4
	—		V_H4-34	GTGTATTACTGTGCGAGAGG		TCCCCTCGGGGGGGTACTTTT	D_H3-16 J_H4
	—		V_H6-1	GTGTATTACTGTGCAAGA GATTGTGCA		AGAGATATGGGGGGGG TACTTTT	D_H3-16 J_H4
	—		V_H4-61	GTGTATTACTGTGC		AGAGAGATGGGGGGGGG TACTTTT	D_H3-16 J_H4
	—		V_H2-70	ACGTATTACTGTGGCACG GATGTGTGCA		CGGATGGGGGAC TACGGGGGGTACTTTG	D_H3-16 J_H4
	—		V_H4-31	GTGTATTACTGTGTGAGA GAA		AGAGAAGGCTACGGGGGG TACTTTT	D_H3-16 J_H4
	—		V_H4-59	GTGTATTACTGTGGCGAGGA TAAA		AGGGACTACGGGGGG TACTTTG	D_H3-16 J_H4
	—		V_H5-51	ATGTATTACTGTGGCGAGC		GAGCCTCTACGGGGGG TACTTTT	D_H3-16 J_H4
BV173[a]	V_H3-38	CGTGTATTACTGTGCCAGA TATA	V_H3-48	GTGTATTACTGTGGCGA	GCCAGATATTGT	AGTGGTGGTAGCT	D_H2-2 J_H3
SUP-B15[a]	V_H3-38	CGTGTATTACTGTGCCAGA TATA	V_H3-53	GTGTATTACTGTGCGAGA	GTTGCCAGGGGG	TGGTGTATGCTATACC	D_H2-8 J_H6
HPB-NULL[b]	V_H3-13	CGTGTATTACTGTGCAAGAGA	V_H4-59	GTGTATTACTGTGCGAGA	CTAAGAGATGG	–	J_H6[c]
KARPAS-422[d]	V_H1-58	TGTGTATTACTGTGCGGCAGA	V_H6-1	GTGTATTACTGTGCAAGAG	TGGGCAGCT	CGTCAAGGGGAGGT	D_H3-22 J_H4

BEL1 and NALM1 pre-B-lymphoblastic leukemia cells exhibit ongoing V_H replacement on one allele (allele 1) and *de novo* V_H to DJ_H rearrangement on the other allele (allele 2). From BEL1 cells, also an *IGHV* germ line allele was amplified (referred to as allele 3). Likely donor–recipient relationships between multiple V_H–DJ_H gene rearrangements were depicted based on the localization of V_H gene segments in the *IGH* locus. cRSS motifs (bold), footprints of recipient V_H gene segments (underlined), [a]V_H gene replacements were already described by Klein *et al*. (2004). [b]The footprint of this potential V_H replacement may also be derived from a V_H3-74 or V_H6-1 gene segment. [c]The D_H gene segment could not be identified. [d]Likely generated by inversion or transrecombination events.

RSS-DNA double-strand break intermediates specific for yet unrearranged J_H5 gene segments were amplified to detect *de novo* D_H to J_H5 rearrangements (Supplementary Figure 1). For detection of secondary rearrangements by V_H replacement, we amplified DNA double-strand break intermediates at the cryptic RSS of an already rearranged V_H gene segment (V_H1-2 in BEL1 cells and V_H6-1 in Karpas-422 cells; Figure 2a; Supplementary Figure 1). To ensure that the amount of target DNA for double-strand breaks was equal, a germline DNA fragment including the J_H5 RSS (*de novo* rearrangements) and the pre-existing VDJ rearrangements (V_H1-2 D_H3-22 J_H6 in BEL1 cells; V_H6-1 D_H3-22 J_H4 in Karpas-422 cells) were amplified. In the case of BEL1 cells, we amplified one germline allele of the *IGHV* locus in addition to two rearranged alleles (Tables 1 and 2), suggesting that this cell line comprises subclones that carry at least one germline allele.

Although DNA double-strand breaks involved in both *de novo* and secondary rearrangements were clearly detectable in SLP65-deficient leukemia and lymphoma cells carrying a green fluorescent protein (GFP)-control vector, reconstitution of SLP65 expression in these cells resulted in a dramatic decrease of the frequency of DNA double-strand breaks (Figure 2a). Likewise, SLP65-deficient leukemia and lymphoma cells carrying only the GFP-control vector express both RAG1 and RAG2, which was sensitive to SLP65 reconstitution in these cells (Figure 2b). We conclude that re-expression of SLP65 in pre-B-lymphoblastic leukemia and lymphoma cells was sufficient to terminate aberrant VDJ recombinase activity. This function of SLP65 may have important implications for the clonal evolution of an SLP65-deficient leukemia or lymphoma because perpetual expression and activity of RAG1 and RAG2 carries the risk of continuous DNA double-strand breaks and

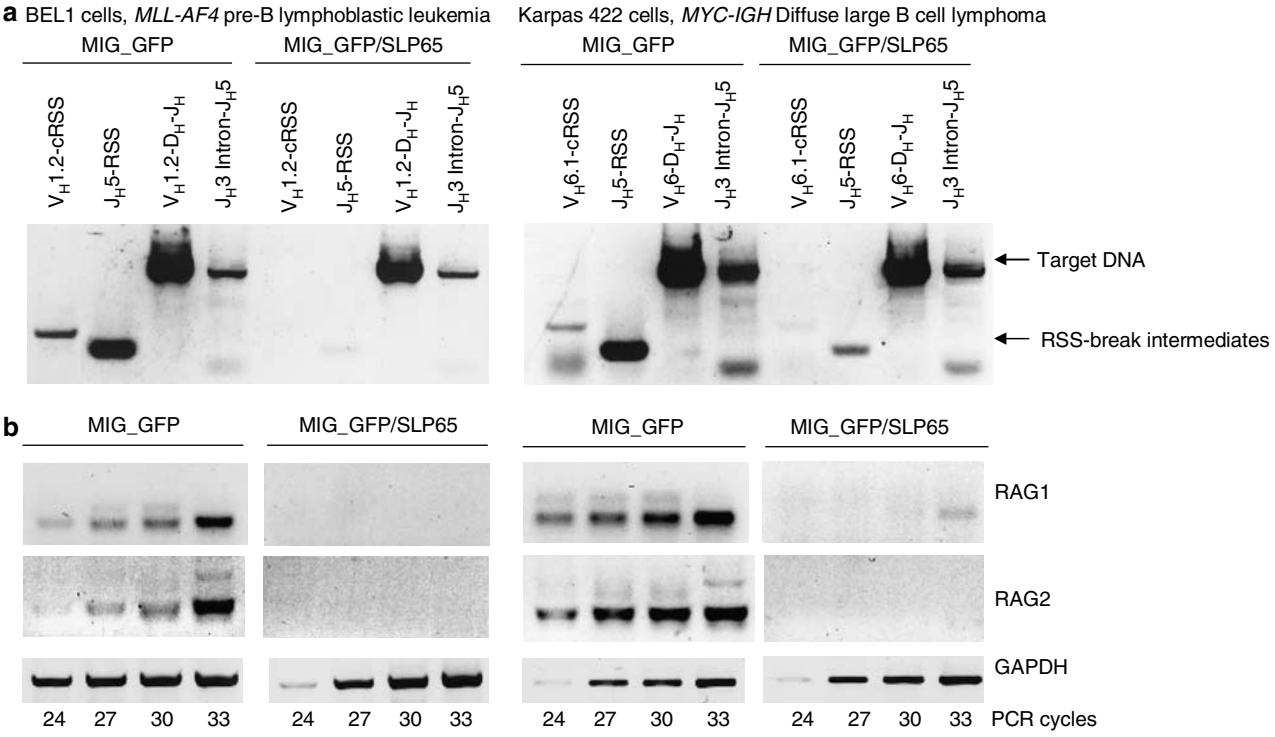

Figure 2 SLP65 deficiency results in perpetual V(D)J recombinase activity in pre-B-acute lymphoblastic leukemia and B-cell lymphoma cells. SLP65-deficient pre-B-lymphoblastic leukemia (BEL1) and lymphoma (Karpas-422) cells were reconstituted with SLP65 by nucleofection according to the manufacturers' protocol (Amaxa Biosystems, Cologne, Germany) using MIG_GFP_IRES_SLP65 and a MIG_GFP vector as a control. The cells were cultured for 24 h and nucleofection was repeated. After 2 days, 5×10^4 GFP-expressing cells were sorted using a FACStar 440 cell sorter and kept under cell culture conditions or subjected to DNA or RNA isolation for ligation-mediated PCR or reverse–transcription PCR analysis, respectively. Short-lived RSS-DNA double-strand break intermediates were determined by ligation-mediated PCR. Target DNA for potential recombination events was amplified as loading control (**a**). Ligation-mediated PCR (LM-PCR) was carried out as described previously (Klein *et al*., 2005) and as outlined in Supplementary Figure 1. In two rounds of semi-nested amplification, DNA intermediates with a double-strand break at the cryptic recombination signal sequence (cRSS) of rearranged V_H gene segments were amplified using the primers listed in Supplementary Table 1. V_H cRSS-specific primers were used together with a primer specific for DNA-ligated linker molecules. To amplify RSS intermediates with a DNA double-strand break at the 5′ heptamer of unrearranged J_H5 gene segments, nested forward primers flanking the J_H5 RSS were used in two rounds of PCR amplification together with a linker-specific primer. To ensure that equivalent amounts of target DNA for potential DNA double-strand breaks by V_H replacement or by *de novo* VDJ rearrangement were present in all LM-PCR reactions, pre-existing V_H1-2 and V_H6-1 gene rearrangements and a genomic region containing the non-rearranged J_H5 gene segment were amplified in one round of PCR. RAG1 and RAG2 expression was analysed by semiquantitative RT–PCR as described previously (Feldhahn *et al*., 2005), using the primer pairs listed in Supplementary Table 1 (**b**). cDNA amounts were normalized by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts using the PCR cycle numbers indicated.

the accumulation of secondary transforming events in the leukemia and lymphoma cells. These findings establish a causative link between perpetual VDJ recombinase activity and SLP65 deficiency not only in pre-B-lymphoblastic leukemia but also in B-cell lymphoma cells.

Abbreviations

DLBCL, diffuse large B-cell lymphoma; *IGH*, immunoglobulin heavy chain; RSS, recombination signal sequence; SLP65, SH2 domain-containing lymphocyte protein of 65 kDa.

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