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μ-Surrogate Light Chain Physicochemical Interactions of the Human PreB Cell Receptor: Implications for V_H Repertoire Selection and Cell Signaling at the PreB Cell Stage[1]

Laurent Gauthier, Bénédicte Lemmers, Valérie Guelpa-Fonlupt, Michel Fougereau, and Claudine Schiff[2]

The surrogate light chain (SL) composed of the λ-like and VpreB polypeptides is organized as two Ig domains and an extra-loop structure. It associates to the μ-chain in preB cells. We have produced human VpreB, SL, two Fdμ (V_H-C_H1), and the two corresponding Fab-like (Fdμ-SL) recombinant proteins in baculovirus. The correctness of the general conformation of the proteins was assessed by epitope mapping and affinity measurements using a new batch of anti-VpreB mAbs. Plasmon resonance analysis showed that both VpreB and the entire SL associated with the Fdμ fragments, with K_d values of 3×10^{-8} M for VpreB-Fdμ and of 10^{-9} to 10^{-10} M, depending upon the V_H, for SL-Fdμ. These results indicate that the λ-like chain, in addition to be covalently bound to the Cμ1 domain, also interacts with the V_H domain. Therefore, a dual role of the SL emerges: 1) interaction of the C-domain of λ-like would release the μ-chain from its interaction with binding protein in the endoplasmic reticulum, and 2) interaction of a part of λ-like and most of VpreB would bind to V_H, ensuring a "quality control" of the native heavy chain that represents the first step of selection of the B cell repertoire. We also demonstrated that two Fab-like fragments did not interact with each other, suggesting that activation of the cell surface preB receptor does not involve aggregation neither in *cis* nor in *trans* of the Fab-like structures. ***The Journal of Immunology*****, 1999, 162: 41–50.**

In bone marrow, proB, and preB precursors that successfully rearrange H and L genes expand and differentiate to give rise to immature B cells. These processes are strictly regulated, and at least two "quality control" checkpoints exist, one dependent on the preB receptor at the transition from large to small preB cells and the second due to the B cell receptor, at the immature B cell stage (1). PreB and B cells are submitted to positive selection for survival, whereas in immature B cells, receptor editing and negative selection occur to eliminate autoreactive B cells (2, 3). Signals from the bone marrow microenvironment can potentially influence each of these checkpoints during lymphocyte development. The $V_H \rightarrow$D-JH rearrangements lead to the preB stage, identifiable by the presence of intracytoplasmic μ-chain (cμ) and/or surface μ-chains, that are associated with the so-called surrogate or pseudo-light chains (SL)[3] (4–6), which is composed of two polypeptides encoded by the VpreB (7, 8) and the λ5 (or λ-like in humans) genes (9–14).

As gene targeting of the λ5 gene (15) as well as that of the membrane exons of the μ-chain (16) block the B cell differentiation at the proB to preB stages, it is clear that the μ-SL complex plays a major role in the clonal expansion of preB cell populations, allelic exclusion (17–19), and repertoire selection (20–23). As a bias in the V_H repertoire is already present at the preB stage, it is clear that the SL chain is of importance in selecting bona fide heavy chains, i.e., μ-chains that have a correct conformation that allows them to further interact with light chains (20). Indeed, recent arguments have been given along this line, as a bias in the expression of the most 3′ V_H gene in preB cells in mice was shown to be strongly dependent upon the presence of the SL (23). However, the precise mode of action of the μ-SL complex has not been elucidated. Although no ligand has been identified to date, several arguments strongly suggest that it has general features of a surface receptor. Thus, stimulation with anti-μ Abs induces intracellular mobilization of calcium and initiates phosphorylation processes in preB cell lines (24–26).

Because control of these early steps of B cell differentiation are strictly dependent upon the expression of the preB cell receptor, physicochemical characteristics of the V_H-Cμ/SL complex should help to elucidate: 1) the successive states of equilibrium that drive the μ-chain from the endoplasmic reticulum to the cell surface, 2) the selection of the correctly folded chains, and 3) the competition with the emerging light chains, which will ensure the preB→B transition. Theoretical models of the μ-SL complex have been proposed (1, 27), based on the fact that λ5/λ-like and VpreB interact with each other and with the heavy chain in a way somewhat similar to a regular Fab. In these models, the λ5 polypeptide contributes the equivalent of the constant light chain domain, a reasonable assumption, based on a 85% homology between λ-like and a regular human Cλ segment, whereas the VpreB, altogether with a short segment of λ-like, may be considered a V_L equivalent domain.

In this paper, we present the first complete analysis of kinetic constants that define the "Fab-like fragment", i.e., the Fdμ-SL complex in its entirety, as it must physiologically interact with the

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[3] Abbreviations used in this paper: SL, surrogate light chain; sc, single-chain; BCR, B cell receptor; aa, amino acid. RU, resonance units; HBS, HEPES buffer saline (10 mM HEPES, 3.4 mM EDTA, and 150 mM NaCl).

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Table I. *Oligonucleotide primers used for PCR amplification*

Primers	5′ to 3′	Underlined	Annealing Temperature (°C)
A	GATCAGATCTATGAAGCTCTTCGTC	*Bgl*II	A + B : 54
B	CCTGCTCTGCGAAGCCAGACACAGTCCAAG	λ-like anti-sense aa 45–48	
C	CTGTGTCTGGCTTCGCAGAGCAGGGCCCTG	λ-like sense aa 45–50	C + D : 55
D	TAGCTGATCATGAACATTCTGCAGGGGC	*Bcl*I	A + D : 54
E	CGCAAAGCTTATGTCCTGGGCTCCTGTC	*Hin*dIII	E + F : 56
F	GCGGAATTCTGATCAAGGGACACGTGTCC	*Eco*RI/*Bcl*I	
G	ATCTCGAGGCGGCCGCATGTCCTGGGCTCCTGTCCTG CTCATGCTGTTTGTCTACTGC	*XhoI*/*Not*I	G + H : 50
H	GCTCTGCGAGGATCCGCCACCGCCAGAGCCACCTCCG CCTGAACCGCCTCCTCCAGGGAACGTGTCCTGGCTGC	anti-sense aa (GSSSS)3	
I	CGTGTCCCTGGAGGAGGCGGTTCAGGCGGAGGTGGC TCTGGCGGTGGCGGATCCTCGCAGAGCAGGGC	sense aa (GSSSS)3	I + J : 50
J	ATGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTC AAGCTTTGAACATTCTGCAGGGGCCACCGTCTTCTC	*Not*I, FlagM2, *Hin*dIII	G + J : 70
L	AAAAGCGGCCGCTTACTTGTCATCGTCGTCCTTGTAGTC AATCACTGGAAGAGGCACGTTC	*Not*I, FlagM2	L + M : 60
M	CCCAAGCTTATGGACTGGACCTGGAGGATC	*Hin*dIII	
N	CCCAAGCTTATGAAACACCTGTGGTTCTTCCTCC	*Hin*dIII	L + N : 60

bone marrow environment during B cell differentiation. For this, we have prepared various combinations of recombinant proteins, expressed in the baculovirus system. Kinetic constants have been determined by the plasmon resonance technique (BIAcore, Saint Quentin-Yvelines, France), whereas the correctness of the general conformation of the SL contributing to the complex was assessed by epitope mapping and affinity using a new batch of mAbs raised against the VpreB polypeptide. Search for potential interactions between two Fab-like complexes was also investigated.

Materials and Methods

Cell lines and culture conditions

JEA 2 (28), BV173 (29), TOM-1 (30), Reh (31), and RS4.11 (32) are proB cell lines and Nalm6 (33) is a preB cell line. Daudi (34) and Jurkat (31) are mature B and T cell lines, respectively. The Abelson-transformed murine proB cell line 38B9 was also used (35). Cell lines were maintained at 37°C in 7% CO_2 in RPMI medium supplemented with penicillin, streptomycin, 10% FCS, and 2 mM L-glutamine.

Cell lysates

Cells (50×10^6/ml) were lysed with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP40.

cDNA preparation

cDNA from preB cell lines was prepared from total RNA of 4×10^6 cells, using 150 ng of oligo dN6 as primer and the reverse transcriptase (Life Technologies, Cergy-Pontoise, France), according to the manufacturer's instructions.

PCR

PCR was performed in a Crocodile III Appligene thermal cycler (Appligene, Illkirch, France) using 1–10 ng of cDNA with 50 pmol of each oligonucleotide as primer (see Table I). The first cycle was run as follows: denaturation at 94°C for 1.5 min, annealing at temperatures indicated in Table I for 2 min, and synthesis at 72°C for 2 min. The next 28 cycles were run similarly except that denaturation lasted only 1 min. For the last cycle, synthesis time was 7 min. Amplified products were run in agarose gel made 2% in 1 × TBE (0.45 M Tris borate pH 8.0, 10mM EDTA) and eluted with Geneclean II (Bio 101, La Jolla, CA).

Construction of the transfer vector containing the native SL chain

Construction of the pUC-FO vector. The pUC-FO vector was derived from pUC19 by inserting, between the *Eco*RI and *Hin*dIII sites, a synthetic DNA fragment containing *Eco*RI, *Hin*dIII, *Bgl*II, and *Bcl*I restriction sites as described in Fig. 1*A*.

Human λ-like signal peptide modification and λ-like cDNA cloning into pUC-FO. The 44-amino acid (aa) λ-like leader peptide (27) was replaced by a 19-aa bovine light chain signal peptide, that was shown earlier to yield efficient production in the baculovirus system (H. Chaabihi, unpublished observations). Bovine light chain signal peptide was first amplified from the plasmid pUCp10PSLTB (Quantum, Montreuil, France,) using primers A and B. Then, λ-like coding segment was amplified from the λ-like cDNA (27) using primers C and D. Finally, the above two PCR products were annealed together and amplified with primers A and D. The resulting PCR product was purified, digested with *Bcl*I and *Bgl*II and ligated into pUC-FO that had been digested with *Bcl*I and *Bgl*II (Fig. 1*A*).

Human VpreB cDNA cloning into pUC-FO λ-like. The complete coding sequence of the human VpreB gene was amplified from the VpreB cDNA (27) with E and F primers. The resulting PCR product was purified, digested with *Eco*RI and *Hin*dIII, and ligated into pUC-FO λ-like vector that had been digested by *Eco*RI and *Hin*dIII (Fig. 1*A*).

Construction of the human SL transfer vector. A head-to-tail tandem P10 baculovirus promotor (supplied by Quantum France) was digested with *Bam*HI and *Hin*dIII and inserted at the *Bgl*II and *Hin*dIII sites of the pUC-FO VpreB-λ-like between the λ-like and the VpreB cDNAs. After digestion with *Bcl*I, the λ-like/tandem P10/VpreB DNA fragment was inserted into pP3ΔEXSp10PATK transfer vector (Quantum France) that had been digested with *Bgl*II to obtain the native SL transfer plasmid (pTB-FoVpreB-λ-like; Fig. 1*A*).

Construction of the transfer vector containing the single-chain SL (scSL)

A complete fusion protein was prepared by inserting a linker made of (Gly-Ser-Ser-Ser-Ser) × 3, designated $(GSSSS)_3$, between the VpreB and the λ-like coding cDNAs and adding an 8-aa (DYKDDDDK) Flag sequence (IBI, Integra Biosciences, Eaubonne, France) at the 3′ end of the λ-like segment. First, the VpreB and λ-like cDNAs were amplified with G and H and with I and J primers, respectively. Then the two PCR products were purified, hybridized, and amplified with G and J primers. The resulting product was purified and cloned into pGEM-T vector (Promega, Charbonnières, France) to obtain pGEM-T-scSL vector. After checking the inserted sequence, the insert-containing *Not*I fragment was ligated into the *Not*I sites of the pGm16 transfer vector (Quantum France) to obtain the pGm16-scSL transfer plasmid (Fig. 1*B*).

Construction of the transfer vectors containing Fdμ (V_H-CH1) chain

The human V_H-CH1 μ-chain-coding regions were amplified from Nalm6 and 1E8 preB cell line RNAs using M and L and N and L primers, respectively. The L primer also contained the Flag sequence. PCR products were purified, digested with *Hin*dIII and *Not*I, and ligated into the pTen21 transfer vector that had been digested with *Hin*dIII and *Not*I, to obtain pTen21-VHCH1-N6 and pTen21-VHCH1–1E8 transfer vectors (Fig. 1*C*).

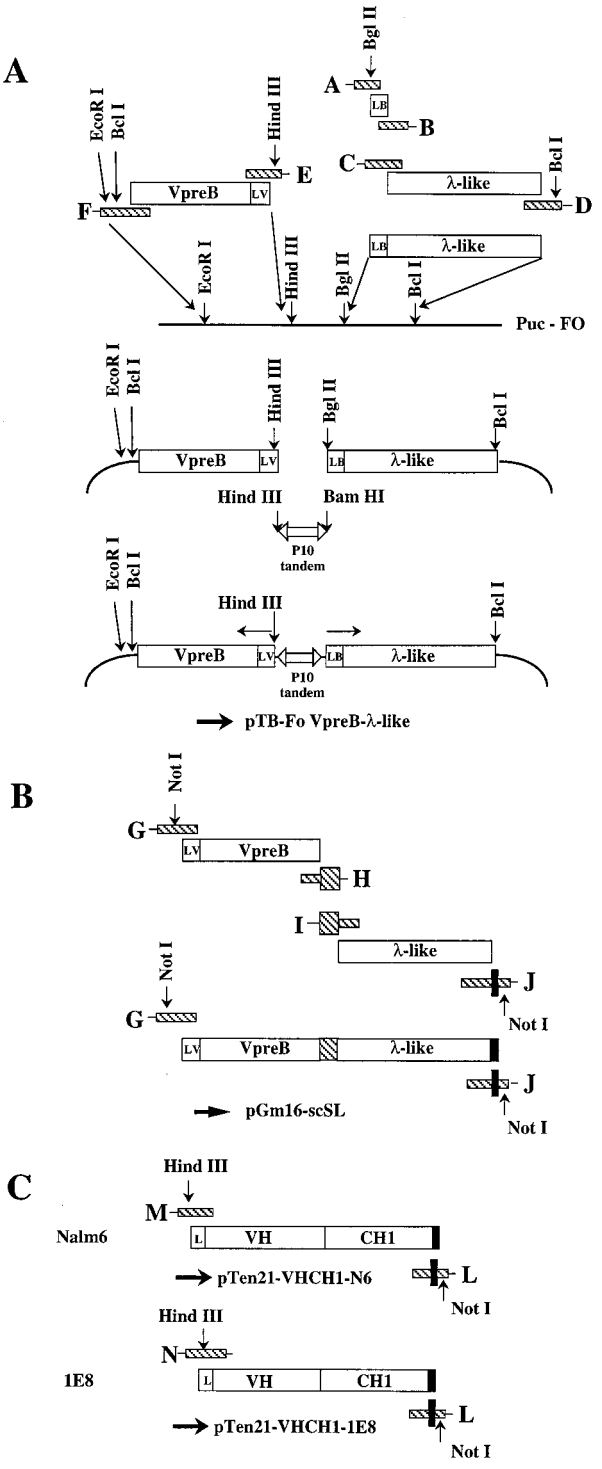

FIGURE 1. Preparation of transfer vectors. *A*, pTB-FO VpreB-λ-like transfer vector used for production of native SL. The vector encodes VpreB with its own signal peptide (LV), a tandem P10 baculovirus promotor (P10), and λ-like with a bovine light chain signal peptide (LB). *B*, construct encoding VpreB, $(GSSSS)_3$ linker peptide, λ-like and a Flag peptide fused together, was ligated into the *Not*I sites of pGm16 vector to yield the pGm16-scSL transfer vector used for production of scSL. *C*, V_HC_H1 μ-Nalm6 and V_HC_H1 μ-1E8 constructs were cloned into the *Hind*III and *Not*I sites of pTen21 vector to obtain pTen21-VHCH1-N6 and pTen21-VHCH1–1E8 transfer vectors used for production of Fdμ Nalm6 and Fdμ 1E8, respectively. Oligonucleotide primers used for PCR amplification (Table I) are indicated by dashed rectangles. Large dashed rectangles correspond to the $(GSSSS)_3$ linker sequence and large closed rectangles to the Flag peptide sequence. Restriction sites are indicated by vertical arrows.

Insect cell culture

Spodoptera frugiperda (SF9) and High Five (Invitrogen, Leek, The Netherlands) insect cells were maintained in adherent cell culture in TC100 medium (Life Technologies, Cergy-Pontoise, France) supplemented with 10% FCS and Excell 401 medium (Valbiotech-Biosciences, Paris, France), respectively.

Production of recombinant viruses

pTB-FO VpreB-λ-like or pGm16-scSL transfer plasmids (5 μg) were cotransfected with linear wild-type baculoviral DNA (0.5 μg) into SF9 cells by using DOTAP cationic liposomes (Boehringer Mannheim, Meylan, France) as instructed by the manufacturer. For pTen21-VHCH1-N6 and pTen21-VHCH1–1E8 transfer plasmids, the same procedure was followed, except that circular wild-type Ac MNPV (36) baculoviral DNA (0.5 μg) was used. Growth, plaque purification, and virus titrations have been already described (37).

Nucleotide sequence analysis

The nucleotide sequence of all constructs have been verified by the dideoxy chain termination protocol with ^{35}SdATP, a modified T7 DNA polymerase, a sequenase kit (United States Biochemical Corporation, Cleveland, OH), using internal VpreB, λ-like (27), and vector-specific primers.

Production and purification of recombinant proteins

Recombinant proteins were produced in adherent High Five cell cultures. Cells ($3–5 \times 10^5$ per ml) were infected with virus at 5–20 infection multiplicity. For soluble (Fdμ-scSL) preBCR production, cells were coinfected with recombinant scSL and V_H-C_H1 chain viruses at an infection multiplicity ratio of 2 and 5, respectively. Culture supernatants were harvested by centrifugation on day 5 or 6 after infection, supplemented with 20 mM sodium phosphate, 150 mM NaCl (final concentrations), pH 7.5, and PMSF protease inhibitor (Sigma, Saint Quentin Fallavier, France).

VpreB containing proteins were purified from culture supernatants by immunoaffinity chromatography using the 4G7 anti-VpreB mAb coupled to Afigel-15 Sepharose (Bio-Rad, Ivry Sur Seine, France) at 6 mg/ml. μ proteins were purified by immunoaffinity chromatography on anti-Flag M2 beads (IBI, Integra Biosciences). Clarified culture supernatants were passed over the column, which was then washed with PBS, pH 7.5, and bound recombinant proteins were eluted with glycine buffer (0.1 mM glycine, pH 3). Eluates were immediately neutralized with 2 M Tris-HCl, pH 8, concentrated with Centricon 30 (Amicon, Beverly, MA) in PBS, pH 8, 0.02% sodium azide, and stored at 4°C. Purified recombinant proteins were quantified by the colorimetric Bio-Rad Bradford Protein Assay (Bio-Rad).

Protein detection analysis

Supernatants and purified recombinant proteins were analyzed by 12% SDS/PAGE followed by Coomassie blue staining.

For Western immunoblotting, proteins were transferred onto 0.2 μm Immobilon-P membranes (Millipore, Bedford, MA) in 20% methanol, 25 mM Tris, and 0.2 M glycine. Membranes were saturated with 5% nonfat dry milk in PBS, 0.05% Tween 20, and incubated with appropriate mouse mAb (anti-VpreB or M2). Revelation was obtained using a goat anti-mouse IgG mAb conjugated to peroxidase (Sigma) followed by chemiluminescence (ECL) (Amersham, Les Ulis, France) detection.

For size purification protein analysis, a 50-μl solution containing 50 μg of protein was loaded onto a Superdex 75 (SMART System, Pharmacia Biotech, Orsay, France) size-exclusion column (3.2 cm × 30 cm) made in PBS, pH 7.4. Fractions (100 μl) were collected and monitored by UV absorbance at 220 nm. To calibrate the size-exclusion column, albumin (molecular weight (M_r) 66,000), OVA (M_r 45,000), chymotrypsinogen A (M_r 25,000), and lysozyme (M_r 14, 300) from Sigma were used as m.w. standards. Recombinant Fdμ homodimers were reduced in 4 mM DTT and alkylated with 8 mM iodoacetamide.

mAbs against the human VpreB protein

mAbs were prepared against the *Escherichia coli* recombinant VpreB protein (28). BALB/c mice were first immunized i.p. with 25 μg of the soluble VpreB protein in CFA and were boosted at wk 2 and 4 with 25 μg protein in IFA. At wk 6, the last injection was performed in PBS. Four days later, spleen cells were fused to the mouse myeloma X63-Ag8.653 using PEG 1500. Hybridoma supernatants were tested on flat-bottom microtiter plates coated with rabbit anti-VpreB Abs (24) onto which the recombinant VpreB protein had been adsorbed. Ten selected hybridomas that recognized the recombinant VpreB protein by Western blotting were cloned by limiting dilution, and Abs were purified from ascitic fluids using protein A adsorbants (B. Lemmers, L. Gauthier, V. Guelpa-Fonlupt, M. Fougereau, and C.

Schiff, manuscript in preparation). Isotypes of the mAbs were determined by an indirect capture ELISA (Southern Biotechnology Associates, Birmingham, AL).

BIAcore analysis

Surface plasmon resonance measurements were performed on a BIAcore apparatus (Pharmacia Biosensor, Saint Quentin-Yvelines, France).

Protein immobilization. Abs or Ags were immobilized covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5 (38). In brief, the sensor chip surface was activated with EDC/NHS (*N*-ethyl-*N′*-(3-dimethylaminopropyl)carbodiimidehydrochloride and *N*-hydroxysuccinimide). Proteins, in coupling buffer (10 mM acetate, pH 5) were injected and deactivation of the remaining activated groups was performed using 100 mM ethanolamine. The immobilized protein surfaces were washed twice with 10 mM NaOH, pH 11, for 1 min to eliminate proteins that were not covalently bound. In all BIAcore experiments, HBS buffer supplemented with 0.05% surfactant P20 served as running buffer.

Affinity measurements. For kinetic measurements, various concentrations of soluble analyte were applied onto the immobilized sample. Measurements were performed at two different continuous flow rates (10 or 20 μl/min) of HBS buffer. The BIAlogue Kinetics Evaluation program (Pharmacia Biosensor) was used for data analysis. For the 4G7 mAb, to check the influence of mass transport effect during the BIAcore measurements, 100 μg/ml of the COOH-terminal VpreB peptide coupled to BSA was injected as a competitor during the dissociation phase.

Results

Production and purification of the human VpreB, scSL, and Fdμ proteins in the baculovirus system

Because the SL chain is formed by the association of the VpreB and λ-like proteins, we first constructed a modified transfer vector in which the complete coding sequence of the VpreB and the λ-like cDNAs were cloned on each side of a head-to-tail tandem P10 promotor as indicated in Fig. 1*A*. The transfer plasmid, pTB-FO VpreB-λ-like, was cotransfected with linear wild-type baculoviral DNA into SF9 cells and three recombinant viruses were isolated. Five days after virus infection of High Five cells, supernatant recombinant proteins were purified by affinity chromatography using the 4G7 anti-VpreB mAb. SDS-PAGE analysis and Coomassie blue staining revealed that the three viruses yielded the same unbalanced pattern of proteins, i.e., a faint amount of λ-like (21 kDa) and an excess of VpreB (16 kDa) proteins (Fig. 2*B*, *panel 1*, *lane 1*). Therefore, this production was used solely as a source for the VpreB purification, which was achieved on Superdex 75 column using the SMART System (Fig. 2*A*, *panel 1*). Fraction 8 with an apparent molecular mass of 38 kDa contained only VpreB dimers as demonstrated by SDS-PAGE analysis (Fig. 2*B*, *panel 1*, *lane 2*). They were present as noncovalent dimers (data not shown).

To obtain the entire SL, we constructed a plasmid transfer vector, pGm16-scSL (Fig. 1*B*) to produce a single chain SL (scSL). After insect cell infection, the recombinant protein was purified on anti-VpreB column and analyzed by SDS-PAGE electrophoresis. As depicted in Fig. 2*B*, *panel 2*, *lane 1*, a major band was observed at 42 kDa, the expected size for the scSL, and a minor band was visible at 20 kDa. This small component was revealed by Western blot analysis, using either an anti-VpreB (Fig. 2*B*, *panel 2*, *lane 2*) or an anti-Flag M2 (Fig. 2*B*, *panel 2*, *lane 3*), suggesting that a partial cleavage had taken place in the vicinity of the linker, leading to free VpreB and Flag-λ-like that remain associated and therefore migrate as the covalently bound scSL on the superdex 75 column (Fig. 2*A*, *panel 2*).

Two V_H-C_H1 μ cDNAs were derived from the human preB cell lines Nalm6 and 1E8, which express the μ-SL complex at the cell surface (24). They were used to construct the two transfer vectors pTen21-VHCH1-N6 and pTen21-VHCH1-1E8 (Fig. 1*C*). After recombinant virus isolation and insect cell infections, recombinant proteins were purified on M2 columns and passed on a superdex 75 column (Fig. 2*A*, *panels 3* and *4*). Materials eluted as one major peak, corresponding to a covalent Fdμ fragment dimer with a molecular mass of 60 kDa or 65 kDa for 1E8 or Nalm6, respectively, as indicated by gel filtration (Fig. 2*A*, *panels 3* and *4*) or by SDS-PAGE analysis (data not shown). After reduction and alkylation, products were analyzed on SDS-PAGE. Coomassie staining and Western blotting identified one single band for the Nalm6 protein (Fig. 2*B*, *panel 3*, *lanes 2* and *4*). For 1E8, two bands were detected by Coomassie staining (Fig. 2*B*, *panel 3*, *lane 1*) from which only the smallest reacted with the M2 mAb upon Western blotting (Fig. 2*B*, *panel 3*, *lane 3*), suggesting the presence of a contaminating protein in addition to the Fdμ fragment. The difference in size observed between the Nalm6 and 1E8 Fdμ fragments was in agreement with their respective lengths (see Fig. 1*C*).

Finally, by coinfection of insect cells with scSL and Fdμ viruses we obtained soluble Fab-like preBCR proteins. After purification on a 4G7 anti-VpreB column, recombinant proteins were analyzed by SDS-PAGE under reducing conditions (Fig. 2*B*, *panel 4*) that revealed the scSL at 42 kDa and Fdμ fragments of 1E8 and Nalm6 at 29 and 32 kDa, respectively. It can be noticed that the contaminant of 1E8 Fdμ at 31 kDa identified in Fig. 2*B*, *panel 3*, *lane 1*, was no longer present in the Fab-like. The minor fraction of cleaved scSL was visible at around 20 kDa (Fig. 2*B*, *panel 4*). When submitted to SDS-PAGE analysis in nonreducing conditions (data not shown), three bands were visible: 1) one major band at 69 kDa (complete covalent structure composed of Fdμ S-S linked to the scSL), 2) one weaker band at about 50 kDa that is not labeled by the 4G7 anti-VpreB Ab and therefore must contain Fdμ S-S linked to λ-like, and 3) a fainter band at 18 kDa that is labeled by 4G7 and is therefore the cleavage product containing VpreB. Taken together with the elution of the major peak on superdex 75 (data not shown), these data point to the presence of two forms of Fab-like, one major in which all elements are covalently linked and one minor in which the VpreB is noncovalently associated.

Characterization of anti-human VpreB mAbs

New mAbs against the human VpreB recombinant protein produced in *E. coli* (28) were prepared as indicated in *Materials and Methods*. Hybridoma supernatants were first tested for their reactivity in ELISA using rabbit anti-VpreB Abs (24) onto which was adsorbed the recombinant VpreB protein (data not shown). After a second screening performed on recombinant VpreB protein by Western blotting, 10 mAbs were selected from which five were used for the present study. All were of the γ1κ isotype, except 15D3 that was γ3κ. Fig. 3 presents the blotting pattern on cell lysates derived from six proB (five human and one mouse) and one preB cells using these mAbs. Two mature cell lines, B (Daudi) and T (Jurkat), served as negative controls. We observed that the five mAbs identified the VpreB protein as a single band except for the RS4.11 cell line for which two bands were detected, as already described (39). The Reh proB cell is negative as expected because no VpreB cDNA may be amplified (data not shown). Moreover, we observed that 4G7/4E7 mAbs gave the strongest signal and that the 14G3 and 15D3 mAbs faintly cross-reacted with the murine VpreB protein.

The overall number of epitopes was first determined by competitive binding of paired mAbs on VpreB and scSL proteins, using the BIAcore system. Four discrete epitopes were identified by 4G7/4E7, 10G5, 14G3, and 15D3 mAbs. Binding inhibition of these mAbs by the 25-aa COOH-terminal peptide of VpreB (40) indicated that 4G7/4E7 recognize the same epitope and that 10G5 defines a closely overlapping epitope on this "extra-loop" portion of VpreB. The two other distinct epitopes were localized on the main domain of VpreB (data not shown).

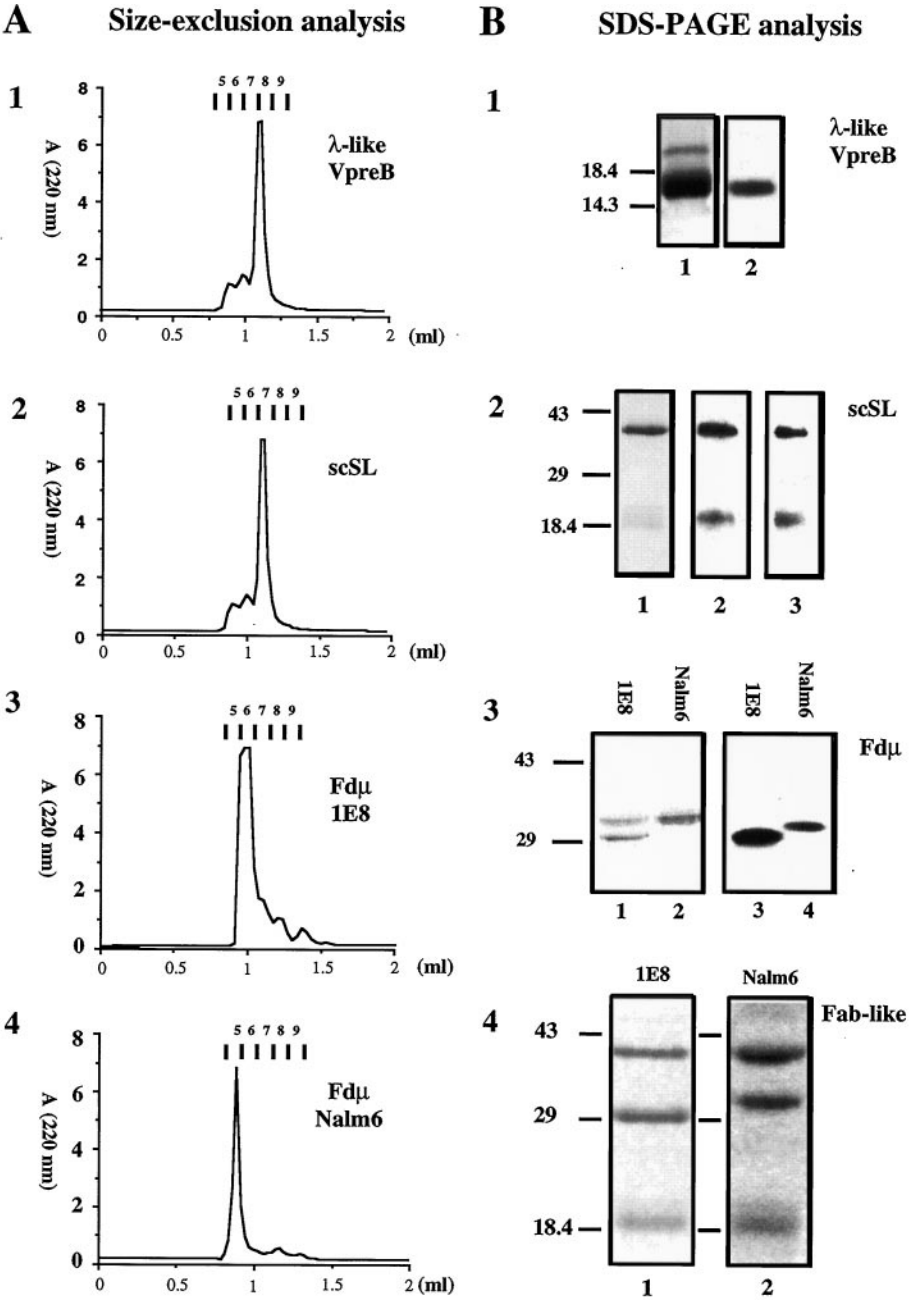

FIGURE 2. Purification of recombinant proteins. *A*, Elution profiles of purified proteins in PBS, pH 7.4, from a Superdex 75 size-exclusion column monitored at 220 nm. The volume of each fraction was 100 μl. The estimated M_r of the eluted samples was determined by reference to standards. Elution volumes of standards were: 0.88 ml for BSA (M_r 66,000), 1.08 ml for OVA (M_r 42,000), 1.26 ml for chimotrypsinogen A (M_r 25,000), and 1.65 ml for lysozyme (M_r 14, 300). *Panel 1*, VpreB and λ-like proteins were copurified from supernatant of insect cells culture infected with the pTB-Fo VpreB-λ-like recombinant virus using 4G7 anti-VpreB mAb. The same purification protocol was applied for scSL (*panel 2*), Fdμ 1E8 (*panel 3*), and Fdμ Nalm6 (*panel 4*), except that pGm16-scSL, pTen21-VHCH1-N6, and pTen21-VHCH1-1E8 recombinant viruses were used, respectively. For the latter two, the M2 anti-Flag mAb was used. *B*, SDS-PAGE analysis. *Panel 1*, Coomassie blue-staining of VpreB and λ-like before loading on Superdex 75 size-exclusion column (*lane 1*) and of the elution fraction 8 (*lane 2*) after SDS-PAGE under reducing conditions. *Panel 2*, Characterization of scSL fraction 7. *Lane 1*, Coomassie blue staining of proteins after SDS-PAGE under reducing conditions. Western immunoblots using 4G7 anti-VpreB (*lane 2*) and M2 anti-Flag (*lane 3*) mAbs (1 μg/ml) were revealed by goat anti-mouse IgG mAb conjugated to peroxidase followed by 5 min chemiluminescence (ECL) detection. *Panel 3*, SDS-PAGE analysis in nonreducing conditions of reduced and alkylated fraction 6 of purified Fdμ 1E8 (*lanes 1* and *3*) and of fraction 5 of Fdμ Nalm6 (*lanes 2* and *4*). Coomassie blue staining was used in *lanes 1* and *2*. Western blot was performed with the M2 anti-Flag mAb in *lanes 3* and *4*. *Panel 4*, Coomassie blue staining of 1E8 (*lane 1*) and Nalm6 (*lane 2*) recombinant Fab-like proteins after SDS-PAGE under reducing conditions.

Conformational analysis of the VpreB, scSL, and the Fab-like preBCR recombinant proteins using the anti-VpreB mAbs

Kinetic analysis of Abs-VpreB epitope interactions was performed using the BIAcore system. As this analysis may be affected by mass transfer effect (41–43), one control was first included for the 4G7 mAb, for which measurements were performed at two flow rates (10 and 20 μl/min) and in the presence of the COOH-terminal VpreB peptide (40) during the dissociation phase. As indicated in Table II, values remained very close whatever the conditions, so that further measurements were performed in one single condition.

Soluble mAbs were passed through the immobilized VpreB, scSL, or Fab-like preBCRs at a continuous flow rate of 10 μl/min. When immobilized VpreB was used, apparent K_d values, calculated from the k_{on} and k_{off} constants, ranged from 4.97×10^{-10} M for the 4G7 to 1.75×10^{-7} M for the 14G3 mAbs (Table III). Whenever VpreB was associated to λ-like, similar values were obtained, indicating that VpreB epitopes were not altered when included in the complete SL. Moreover, constants remained unaffected when the scSL chain was associated to the Fdμ fragment, except for 15D3, in which case expression of the epitope was abolished (Table III). Because we have also demonstrated the presence of four different VpreB epitopes, upon which two are localized on the 25-aa COOH-terminal VpreB peptide, these data indicate that the new mAbs are good sensors of the integrity of the different recombinant proteins.

Kinetic analysis of VpreB, scSL, and Fdμ interactions within the Fab-like fragment

Recovery of the native conformation in recombinant proteins prompted the analysis of physicochemical constants of the various partners that ultimately form the "surrogate" or Fab-like structure. These measurements were also made by the plasmon resonance method. It was first shown that VpreB interacted similarly with itself or with scSL, as indicated from the K_d values of the order of

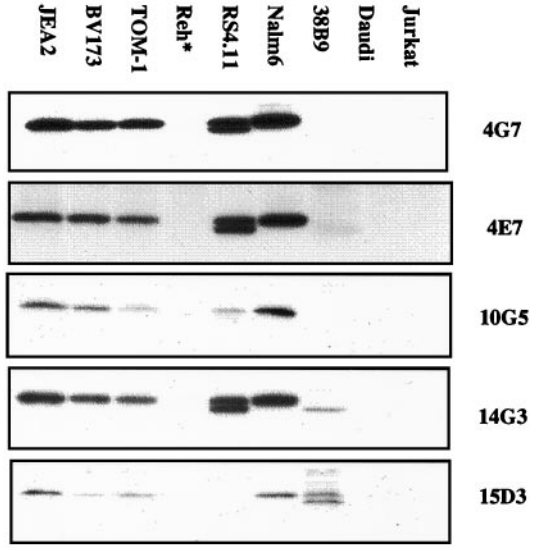

FIGURE 3. Western immunoblotting of total cell lysates from different human or mouse cell lines using anti-VpreB mAbs. Cells were lysed with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP40. For each lysate, 10 μg of total proteins were loaded onto the gel. Western immunoblotting with anti-VpreB mAbs (4G7, 4E7, 10G5, 14G3, and 15D3 as indicated) was performed as described in Fig. 2. The cell lines were as follows: JEA2, BV173, TOM-1, Reh, and RS4.11 (human proB), 38B9 (mouse proB), Nalm6 (human preB), Daudi (human B), and Jurkat (human T). $*$, The proB cell line Reh used in this experiment does not express VpreB transcripts.

10^{-8} M (Table IV). Usage of a complete scSL yielded a 10-fold increment in the K_d constant. These values were clearly very close to those from constants measured for the interactions of VpreB and/or scSL with the heavy chain V_H-C_H1 domains, pointing to a similar type of interactions in the SL-SL homodimers and SL-H heterodimers. Values were roughly similar whenever different heavy chain partners were used, although a higher affinity was observed with 1E8 as compared with the Nalm6 heavy chain.

Search for Fab-like/Fab-like interactions

Because *cis* interactions of the preB receptor have been invoked as responsible for initiation of the transduction cascade in preB cells (44, 45), the search for Fab-like/Fab-like interactions was done using either the BIAcore system or the FACS analysis. When Fab-like Nalm6 was immobilized on sensor chip, neither itself nor Fab-like 1E8 interacted, the 4G7 mAb being used as a positive control (Fig. 4*A*). Similar results were obtained whenever 1E8 was immobilized (data not shown).

FACS analysis led to the same conclusions, as the preB complex, revealed by anti-μ and 4G7 anti-VpreB mAbs, was present at the cell surface of Nalm6 and 1E8 (Fig. 4*B*, *top*) and did not allow the fixation of neither Fab-like proteins (Fig. 4*B*, *bottom*).

Coinfection of native SL and 1E8 Fdμ virus yields sufficient native Fab-like proteins to confirm the above BIAcore and FACS results (data not shown), suggesting that the usage of a scSL is not responsible for the lack of Fab-like/Fab-like interactions.

Discussion

On the grounds of homology between the SL and the regular Igλ chain, it may be anticipated that the general structure of the preB receptor, and thus that of its Fab-like equivalent, closely resembles that of their Ig counterparts, with major interactions occurring between λ-like and Cμ1 on the one hand, and between VpreB and V_H on the other hand. In addition, it was also proposed (27) that the COOH-terminal region of VpreB (20 aa) and the NH2-terminal portion of λ-like (50 aa) were looping out from the main domain structure. In this paper, we report the reconstruction, epitopic mapping, and physicochemical characteristics of the Fab-like portion of the preB receptor, which support the previous model and provides additional basis for the role of SL in selection of the preB μ repertoire. We also show that the Fab-like structure does not dimerize, which precludes that signaling at the preB stage operates through homophilic interactions involving the Fab-like structures.

The different components of the Fab-like portion of the preB receptor were obtained as recombinant proteins produced in the baculovirus system. Free VpreB was derived from a construction in which unbalanced synthesis between λ-like and VpreB ensured enrichment of this polypeptide. To obtain the entire SL protein, we made a construct in which the two λ-like and VpreB cDNAs were covalently joined by a linker (scSL). Finally, we produced V_H-C_H1 (Fdμ) constructs from two μ cDNAs cloned from the Nalm6 and 1E8 preB cell lines. Coinfection of scSL and Fdμ viruses yielded Fab-like proteins in which both polypeptides were disulfide linked. For analysis with the BIAcore device, low amount of VpreB protein was immobilized and washed with NaOH, pH 11, to minimize contribution of immobilized dimers.

Using these materials, we first reported the characteristics of five mAbs prepared against the VpreB recombinant protein, which were selected for their ability to immunoprecipitate the VpreB protein, retain binding in Western blot, and pertain to a γ,κ isotype. Binding constants, derived from kinetics measurement using the BIAcore technology, gave K_d values comprised between 10^{-7} and 10^{-10} M, indicative of a correct folding of all constructs (see Table III). Four distinct epitopes were identified (Fig. 5), two in the Ig-like domain and two in the COOH-terminal region of VpreB ("extra-loop"), which cannot be accommodated within the Ig domain structure (27). Most epitopes remained identifiable whatever the construction, i.e., VpreB, scSL, or Fab-like, but one, 15D3, that was lost upon association to the μ-chain counterpart. Conservation of the two extra-loop epitopes in all associations suggests that this

Table II. *Rate and affinity constants of 4G7 mAb to VpreB under different conditions*[a]

Flow (μl/mn)	Competitor During Dissociation Phase	k_{on}[b] ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	$K_d = k_{off}/k_{on}$ (M)
10	none	2.55×10^5	1.26×10^{-4}	4.97×10^{-10}
20	none	1.83×10^5	0.81×10^{-4}	4.43×10^{-10}
10	VpreB peptide-BSA[c]	2.55×10^5	2.06×10^{-4}	8.06×10^{-10}

[a] Six serial dilutions (200:6.25 nM) of 4G7 mAb were injected at different flow rates on a surface containing 700 RU of VpreB. The BIAcore Kinetics Evaluation program was used for data analysis.

[b] k_{on}, association rate constant; k_{off}, dissociation rate constant; $K_d = k_{off}/k_{on}$, apparent dissociation constant.

[c] COOH-terminal VpreB peptide coupled to BSA, used as a competitor (100 μg/ml in HBS buffer) was injected during the dissociation phase.

Table III. *Rate and affinity constants of anti-VpreB mAbs to different recombinant proteins coupled to the dextran matrix*[a]

Soluble mAbs	Immobilized VpreB			Immobilized scSL			Immobilized Fab-like Nalm6			Immobilized Fab-like 1E8		
	k_{on}[b]	k_{off}	K_d	k_{on}	k_{off}	K_d	k_{on}	k_{off}	K_d	k_{on}	k_{off}	K_d
4G7	2.55×10^5	1.26×10^{-4}	4.97×10^{-10}	2.80×10^5	1.93×10^{-4}	4.79×10^{-10}	8.26×10^4	3.52×10^{-5}	4.27×10^{-10}	1.66×10^5	1.05×10^{-4}	6.30×10^{-10}
4E7	3.78×10^5	3.98×10^{-5}	1.05×10^{-10}	1.36×10^5	3.01×10^{-5}	1.01×10^{-10}	ND	ND	ND	ND	ND	ND
10G5	1.97×10^5	6.13×10^{-4}	3.11×10^{-9}	4.70×10^5	1.98×10^{-4}	4.20×10^{-10}	2.15×10^5	5.69×10^{-4}	2.65×10^{-9}	2.09×10^5	1.29×10^{-3}	6.20×10^{-9}
14G3	8.46×10^3	1.48×10^{-3}	1.75×10^{-7}	9.65×10^4	1.70×10^{-3}	1.77×10^{-8}	3.53×10^3	1.33×10^{-3}	3.77×10^{-7}	2.07×10^3	1.26×10^{-3}	6.08×10^{-7}
15D3	1.10×10^5	2.55×10^{-3}	2.31×10^{-8}	7.05×10^4	1.02×10^{-3}	1.45×10^{-8}		no binding			no binding	

[a] Six serial dilutions of mAb, 200:6.25 nM for 4G7, 4E7, and 10G5 and 800:25 nM for 15D3 and 14G3 were injected on four surfaces containing 700 RU, 410 RU, 1400 RU, and 900 RU of VpreB, scSL, Fab-like Nalm6 preBCR, and Fab-like 1E8 preBCR, respectively. The BIAcor Kinetics Evaluation program was used for data analysis.

[b] k_{on}, association rate constant ($M^{-1} \cdot s^{-1}$); k_{off}, dissociation rate constant (s^{-1}); $K_d = k_{off}/k_{on}$, apparent dissociation constant (M); ND, not determined.

Table IV. *Rate and affinity constants of VpreB and scSL to different proteins coupled to the dextran matrix*[a]

Soluble Analyte	Immobilized VpreB			Immobilized scSL			Immobilized V_H-C_H1 Nalm6			Immobilized V_H-C_H1 1E8		
	k_{on}[b]	k_{off}	K_d	k_{on}	k_{off}	K_d	k_{on}	k_{off}	K_d	k_{on}	k_{off}	K_d
VpreB	4.29×10^4	1.08×10^{-3}	2.52×10^{-8}	3.10×10^4	0.99×10^{-3}	3.20×10^{-8}	3.60×10^4	1.14×10^{-3}	3.16×10^{-8}	4.57×10^4	1.15×10^{-3}	2.51×10^{-8}
scSL	3.82×10^4	3.03×10^{-4}	7.90×10^{-8}	3.64×10^5	5.54×10^{-4}	1.52×10^{-9}	3.45×10^5	4.10×10^{-4}	1.19×10^{-9}	3.12×10^5	8.38×10^{-5}	2.68×10^{-10}

[a] Six serial dilutions (400:12.5 nM) of VpreB or scSL were injected over four surfaces containing 700 RU, 410 RU, 870 RU, and 970 RU of VpreB, scSL, V_H-C_H1 Nalm6, and V_H-C_H1 1E8, respectively. The BIAcore Kinetics Evaluation program was used for data analysis.

[b] k_{on}, association rate constant ($M^{-1}s^{-1}$); k_{off}, dissociation rate constant(s^{-1}); $K_d = k_{off}/k_{on}$, apparent dissociation constant (M).

FIGURE 4. Search for Fab-like/Fab-like interactions. *A*, BIAcore analysis of Fab-like/Fab-like interactions. In separate experiments, 20 μl (50 μg/ml) of either 4G7 anti-VpreB mAb, Fab-like Nalm6 preBCR, or Fab-like 1E8 preBCR were injected at a flow rate of 5 μl/min in HBS buffer on a surface containing 1400 RU of Fab-like Nalm6 preBCR. Resulting sensorgrams are superimposed. *B*, Flow cytometric analysis of Nalm6 and 1E8 human preB cell lines surface staining with anti-μ and 4G7 anti-VpreB mAbs, Fab-like Nalm6, and Fab-like 1E8 preBCRs (solid FACS display). Fab-like Nalm6 preBCR and Fab-like 1E8 preBCR staining was revealed with M2 anti-Flag mAb. Unstained cells are also shown (open FACS display).

region of the VpreB chain remains accessible in the complete molecule. Needless to say that these "extra-loop " epitopes, which are not hindered upon association with μ, are highly suitable to investigate expression of the SL at earlier stages of B cell differentiation, before IgH gene rearrangement (B. Lemmers, L. Gauthier,

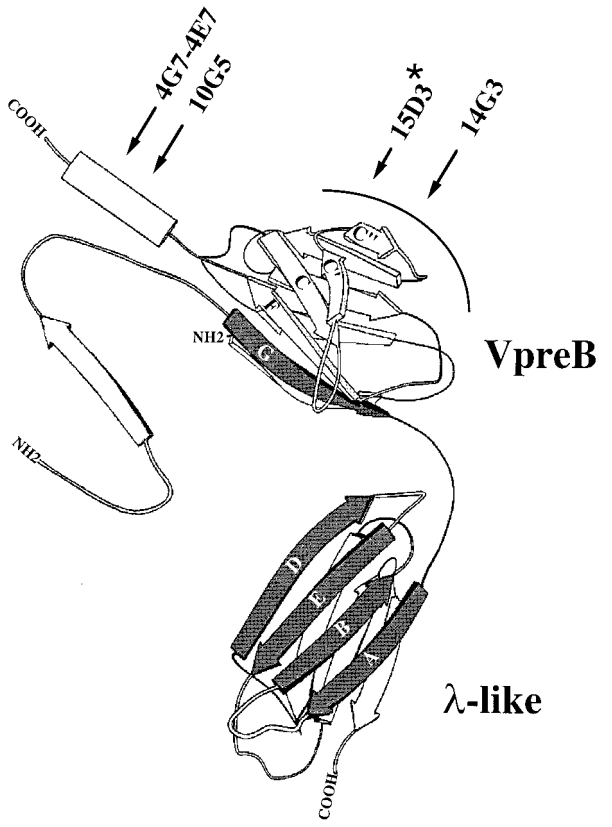

FIGURE 5. Three-dimensional schematic representation of the human SL showing VpreB epitopes and SL β-strands that may interact with Fdμ chain. On the three-dimensional model of the human SL (27), β-strands that may interact with Fdμ are A, B, D, E, and G for λ-like (dark gray) and C, C′, C″, and F for VpreB (light gray). The presumed localization of the different VpreB epitopes are indicated by arrows. ∗, Indicates that the 15D3 epitope is lost when SL interact with Fdμ.

V. Guelpa-Fonlupt, M. Fougereau, and C. Schiff, manuscript in preparation).

Kinetics measurement of the association of the various partners of the Fab-like proteins revealed interesting features (Table IV). It was first observed that VpreB could form homodimers. In addition, kinetics constants were almost identical for association of VpreB with the complete SL or with either heavy chain fragments, with K_d values between 2 and 3×10^{-8} M. These values differed by almost one order of magnitude from those reported for the VpreB-V_H interactions by Hirabayashi et al. (46), which were between 4 and 7×10^{-7} M. The lower affinity observed by the later group may have been due to the dimerized state of the VpreB or V_H preparations as the range of RU that was used was high. In addition, the VpreB molecule used by the authors lacked the 6 COOH-terminal amino acids, which may have affected its physicochemical characteristics. At this point, our data indicate 1) that VpreB may form homo- or heterodimers, 2) that accessibility to VpreB has remained intact in the complete SL, and finally 3) that this situation is totally reminiscent of that of the Ig light chains that may form homodimers in the absence of heavy chains or Ig heavy-Ig light chain heterodimers in Igs.

The entire scSL had a greater affinity than VpreB for the Fdμ fragment (Table IV). In addition, the discrete behavior of VpreB vs scSL regarding binding characteristics to two distinct Fdμ fragments is quite remarkable. Affinity of scSL differed by almost one order of magnitude when binding to Nalm6 or 1E8 (K_d 1.19×10^{-9} M and 2.68×10^{-10} M, respectively), whereas it was the same for VpreB (K_d 3.16×10^{-8} M and 2.51×10^{-8} M, respectively). These values clearly indicate that 1) the complete SL interacts with the heavy chain in the same range of affinities that was reported earlier for heavy and light chain interactions (47, 48) and that 2) the λ-like contribute significantly to selective binding of various V_H regions. If one considers the theoretical model proposed for the SL (27) and, hence, for the Fab-like fragment (Fig. 5), it is clear that interaction with C_H1 must solely rely on λ-like (β strands A, B, D, and E), whereas interaction with V_H, although major with VpreB, also involves binding to the "J-equivalent" portion of λ-like (β strand G). Interestingly, this β-strand has also been proposed to interact with VpreB in the V-like domain (27). This section represents one of the five major β-strands of interactions between H and L within the V domains, the remaining four coming likely from VpreB (β strands C, C′, C", and F). On these grounds, one may consider that the SL has a dual role toward the heavy chain expression in preB cells: 1) interaction of the C domain of λ-like would release the heavy chain from its interaction with binding protein in the endoplasmic reticulum and 2) interaction of a part of λ-like and most of VpreB would bind to V_H, ensuring a first "quality control" of the native heavy chain, and thus a first step of selection of the B cell repertoire, as previously suggested (20–22). In a recent paper, dissection of the rearranged genes at the single-cell level in $\lambda5^{-/-}$ mice confirmed the importance of SL in selecting the VH repertoire (23). In addition, transfection experiments clearly showed that 1) absence of certain VH segments, such as VH81X, at the surface of preB cells was due to their physical inability to associate with SL (20, 23), and 2) cells devoid of preB cell receptor were eliminated, thus raising a possible role, although as yet not proven, of this receptor at the cell surface.

At the preB→B transition, a transient coexpression of SL and light chain must take place, during which the efficiency of Ig light chain to compete with SL for binding to the heavy chain will depend on the relative affinity constant values and concentrations of both light chain and SL partners. Down regulation of SL synthesis will obviously favor heavy and light chain pairing and IgM expression, which is known to take place at the large preB→small preB cells transition (49). In addition, this step of selection will favor formation of heavy and light chain pairs with a higher affinity, which may account for the existence of preferential associations of individual heavy and light chains reported long ago (50). Light chains having the lowest affinity binding for the heavy chains would be negatively selected. An intermediate situation in which affinities and/or concentration of light chain and SL would be rather close might account for the existence of cells producing both the preB and B receptors, as is the case for 1E8 cell line, for which the affinity of SL was particularly high (see Table IV). In this regard, it might be interesting to compare the respective affinities of κ and SL for the Fdμ of the 1E8 cell line.

Several reports have questioned the role of SL in controlling the differentiation of the B lineage on the ground of experimental systems in which expression of the preB receptor was deeply affected, if not completely abolished. Gene targeting of λ5 severely impairs the preB→B transition, but the mutation remains leaky, with a progressive recovery of mature B cells after several months (15). Selection of cells that may have rearranged the IGK locus before IgH has been proposed as a possible explanation (51). An alternative might also be that a μ-VpreB complex could form and escape retention from the endoplasmic reticulum and be expressed at the cell surface. The high affinity of interactions between Fdμ and VpreB that we have reported (Table IV) clearly indicates that such complexes may be formed.

What triggers the preB receptor remains a major unanswered question, and identification of a potential ligand is still awaited. An alternative would be that signaling from the preBCR initiates from *cis* or *trans* homoligation of the receptor. Such an hypothesis has been proposed on the grounds that transgenic mice expressing ΔV-μ-chains in a SCID background could proceed to the preB stage (45) due to the aggregation of the truncated heavy chain at the cell surface. Another report, in which transgenic mice expressing heavy chain deleted from their V_H and C_H1 domains in a $RAG^{-/-}$ background (52) led to somewhat similar conclusions. As we provide evidence that neither homodimerization nor heterodimerization of the Fab-like fragments did occur, self-aggregation of truncated receptor would rather appear as artifactual, whereas in a physiological situation it would be prevented by the presence of the Fab-like at the cell surface. Therefore, activation of the preB receptor by an external ligand remains plausible. This hypothesis was recently discussed by Pelanda et al. (3), who considered that the preB cell receptor has evolved as a "surrogate autoreactive BCR." Whether the ligand should be considered monomorphic or polymorphic remains entirely open to question. Whatever the potential of the μ-SL complex to be a cell surface receptor, it seems clear that its molecular organization is completely compatible with the dual role of SL: 1) to release the μ-chain from its interaction with binding protein in the endoplasmic reticulum and 2) to select those heavy chains with a correct conformation that will ultimately allow them to pair with regular Ig light chains, which implies a role in selecting a "preB repertoire" (20).

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