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*J Immunol* 2001; 167:6403-6411; doi: 10.4049/jimmunol.167.11.6403

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Surrogate Light Chain-Mediated Interaction of a Soluble Pre-B Cell Receptor with Adherent Cell Lines

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Signals initiated by the precursor B cell receptor (pre-BCR) are critical for B cell progenitors to mature into precursor B cells. The pre-BCR consists of a homodimer of μH chains, the covalently associated surrogate light L chain (SL chain) composed of VpreB and A5, and the transmembrane signal molecules Igα and Igβ. One way to explain how maturation signals are initiated in late progenitor B cells is that the pre-BCR is transported to the cell surface and interacts from there with a ligand on stroma cells. To address this hypothesis, we first produced soluble Fab-like pre-BCR and BCR fragments, as well as SL chain, in baculovirus-infected insect cells. Flow cytometry revealed that, in contrast to Fab-like BCR fragments, the soluble pre-BCR binds to the surface of stroma and several other adherent cell lines, but not to B and T lymphoid suspension cells. The specific binding of the soluble pre-BCR to stroma cells is saturable, sensitive to trypsin digestion, and not dependent on bivalent cations. The binding of pre-BCR seems to be independent of the H chain of IgM (μH chain), because SL chain alone was able to interact with stroma cells. Finally, soluble pre-BCR specifically precipitated a 135-kDa protein from ST2 cells. These findings not only demonstrate for the first time the capacity of a pre-BCR to specifically bind to a structure on the surface of adherent cells, but also suggest that the pre-BCR interacts via its SL chain with a putative ligand on stroma cells. The Journal of Immunology, 2001, 167: 6403–6411.

Human as well as murine B lymphocytes develop after birth from pluripotent hemopoietic stem cells in the bone marrow. The growth, differentiation, and survival of B-lineage cells are controlled at different stages of development by cell-cell interactions between B lymphoid precursors and the bone marrow microenvironment; the availability of cytokines, chemokines, and hormones (reviewed in Refs. 1, 2, and 3); and the presence of functional Ig receptors (reviewed in Refs. 4–8).

The development of mature B lymphocytes from hemopoietic stem cells is characterized by stepwise and stage-specific DNA rearrangements of Ig V, (D), and J gene segments, resulting in the generation of Ig genes encoding IgH and IgL chains (reviewed in Ref. 9). V(D)J rearrangement begins at the H chain locus in early progenitor B (pro-B) cells. Pro-B cells harboring a functional V(D)J exon synthesize the transmembrane form of a functional IgH chain of the μ-isotype (μH chain) and develop first into large early precursor B (pre-B) cells and finally into small pre-B cells, in which rearrangement at the IgL locus begins (10). Once a small pre-B cell produces an L chain able to pair with μH chain, it will develop into a surface IgM-positive immature B cell. Besides generating functional Ig genes, V(D)J recombination can also give rise to either nonproductive Ig genes (9) or genes that, for example, encode μH chains unable to pair with conventional IgL chains (11, 12). Therefore, checkpoints must exist at which B lymphoid cells are screened for the presence of functional Ig chains, i.e., those able to form a functional Ag receptor.

An early checkpoint has been identified at the transition from the late pro-B to the early pre-B cell stage, at which B-lineage cells are screened for the presence of a functional membrane-bound μH chain, that is, one capable of forming the so-called pre-B cell receptor (pre-BCR) with an IgL-like surrogate L (SL) chain (13–16) and the signal transducer Igαβ complex (pre-BCR) (Fig. 1) (reviewed in Ref. 17). The SL chain is composed of the two noncovalently associated invariant Ig-like polypeptides VpreB and A5 (reviewed in Ref. 17). Both VpreB and A5 are encoded by genes that do not undergo gene rearrangement and are expressed in early pro-B cells before V(D)J recombination begins (18). Computer modeling, using as reference the structure of a conventional L chain, revealed that VpreB contains one V-like and A5 one C-like Ig fold domain (18, 19). In addition to Ig-like sequences, VpreB and A5 contain at their C-terminal and N-terminal end, respectively, a unique tail without sequence similarities to any known protein. When compared with the number of β-strands in Ig domains of a conventional IgL chain, VpreB lacks the seventh β-strand (β7) of a typical V L region and A5 contains, in addition to all β-strands found in a conventional C region, one extra β-strand. The extra β-strand in A5 seems to replace the missing β-strand in VpreB, since this extra β-strand in A5 is required for the noncovalent association of A5 with VpreB (20).

Experiments with transgenic and gene-targeted mice clearly showed the importance of the pre-BCR for early B cell development (4, 5, 7, 8). For example, the progression of B-lineage cells from the late pro-B to the small pre-B cell stage is severely compromised in Igβ (21)-, VpreB1/2 (22)-, A5 (23)-, and μm-deficient mice (24). Furthermore, full-length μH chains that fail to assemble with either SL chain (11) or the B lymphoid-specific signal-transducing Igαβ complex (25) do not rescue B cell development in transgenic mice unable to rearrange their endogenous IgH gene segments. Pre-BCR-mediated signals have also been implicated in the clonal expansion of early pre-B cells synthesizing a functional μH chain (26, 27), the termination of further rearrangements at the...
Furthermore, this binding does not seem to rely on the presence of lines, but not to several B and T lymphoid suspension cell lines.

**Materials and Methods**

**Cell lines and culture conditions**

The surface IgM-positive mouse B cell lines NYC (35) and WEHI231 (36), the Abelson leukemia virus-transformed mouse pre-B cell lines TK and TK μ (37), the mouse plasmacytoma J558L (38), the mouse T cell lines EL-4 (ATCC TIB-39; American Type Culture Collection, Manassas, VA) and DO11.10, the mouse stroma lines ST2 (39) and PA6 (40), the mouse fibroblast cell line NIH3T3 (41), the human embryonic kidney line 293T (42), the human cervix carcinoma line HeLa (ATCC CCL-2), the human epidermal larynx carcinoma line HEP-2 (CCL-23; American Type Culture Collection), the human T cell leukemia Jurkat (ATCC TIB-152), and the Chinese hamster ovary line CHO (ATCC CCL-61) were all maintained at 37°C in 5% CO₂ in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 5% FCS, 1 mM sodium pyruvate, and 2 mM l-glutamine. *Spodoptera frugiperda* insect cells (IPLB-SF21-AE) (43) were maintained as adherent cells in TC100 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin.

**Antisera and mAbs**

Rat IgM mAbs directed against mouse VpreB (clone VP245) (44), λ5 (clone LM34) (44), and the pre-BCR (clone SL156) (44), the monoclonal hamster anti-mouse A5 Ab, FS1 (37, 45), and unpurified total anti-VpreB rabbit serum generated against a GST-mouse VpreB-fusion protein were previously described (37). FITC-conjugated as well as unconjugated affinity-purified goat Abs against mouse μ- and κ-chain were purchased from Southern Biotechnology Associates (Birmingham, AL); the mouse IgG anti-penta-His mAb from Qiagen (Hilden, Germany); the FITC-conjugated goat F(ab’)_2 anti-mouse IgG (H and L chains), goat anti-rat IgC (Fcγ specific), rabbit anti-goat IgG (H and L chains), and the HRP-conjugated rabbit anti-hamster IgG Abs from Dianova (Hamburg, Germany); and the HRP-conjugated goat anti-rabbit IgG (H and L chains) and goat anti-mouse IgG (H and L chains) Abs from Bio-Rad (München, Germany).

**Construction of baculovirus transfer vectors**

DNA fragments encoding the mature form of mouse VpreB, λ5, κL chain as well as the V (VH) and first C (Cμ1) domain of the μ chain were amplified by PCR from plasmids containing the corresponding cDNA sequences with the following primers: for VpreB, 5’-TAGGGCTTACCATGAA GGTTCGTGAAAGCTGAGCAGGAAATGTCGATC-3’ and 5’-TCTAG AGAGAGGCTGAATATAGATTTCATCCAGATGC-3’ for the μ chain, 5’-TCTAG AGAGAGGCTGAATATAGATTTCATCCAGATGC-3’ and 5’-TCTAGGCTTACCATGAA GGTTCGTGAAAGCTGAGCAGGAAATGTCGATC-3’ for the κL chain. PCR products were purified, ligated into the pCR2.1 cloning vector (Invitrogen, Groningen, The Netherlands), and verified by DNA sequencing using the Big-Dye sequencing kit from PerkinElmer Biosystems (Warrington, U.K.). A *Smal*/XhoI λL chain fragment was isolated from the respective pCR2.1 vector and ligated into the transfer vector pFASTDual (Life Technologies). An Fdμ fragment was isolated as a *BamH*I/XhoI fragment and ligated into both the transfer vector pFASTBac1 (Life Technologies) and pFASTDual-κL vector. A *Sal*I/Adv fragment was ligated from the pCR2.1 cloning vector into the pCR2.1 cloning vector (Invitrogen, Groningen, The Netherlands), and verified by DNA sequencing using the Big-Dye sequencing kit from PerkinElmer Biosystems (Warrington, U.K.). A *Sal*I/Adv κL chain fragment was isolated from the respective pCR2.1 vector and ligated into the transfer vector pFASTDual. The transfer vector pFASTDual-VpreB/A5 was constructed by inserting a *Sal*I fragment from the respective pCR2.1 vector into the pFASTDual-VpreB transfer vector. To assemble a vector encoding VpreB, A5, and Fdμ (pFAST-VpreB/A5/Fdμ), a *Sal*I/Aval fragment containing the SV40 poly(A) site and the melittin-Fdμ sequence under the control of the polyhedrin promoter was isolated from pFASTBac1-Fdμ, blunt ended with Klenow enzyme, and ligated into the *Sal*I site of the pFASTDual-VpreB/A5 transfer vector.

**FIGURE 1.** Hypothetical domain structure of the pre-BCR complex. Both VpreB (light gray) and λ5 (dark gray) contain Ig-like domains (indicated by loops) and non-Ig tails (marked with dotted lines) at the C- and N-terminal end, respectively (17). Both chains are noncovalently associated via the seventh β-strand of λ5 (indicated by a black frame and marked with a black arrow) to form the SL chain (20). SL chain is disulfide linked with μ chain via the penultimate cysteine of λ5 and probably a cysteine of μC1, the first C domain of the μH chain. Disulfide bonds are indicated by thick black lines, membrane regions by gray-shaded cylinders, Ig fold domains by loops, and cytoplasmic tails of μH chain and Igα/β by open rectangles.

IgH locus (28–30), and the redirection of the V(D)J recombinase from the IgH to the IgL locus in late pre-B cells (31, 32).

Although it is generally accepted that signals initiated by a functional pre-BCR are critical for B-lineage cells to efficiently pass the checkpoint between the late pro-B and early pre-B stage (reviewed in Ref. 33), little is known about the mode of signaling of this receptor. Several models to explain how pre-BCR-mediated signals are initiated are discussed in the literature (reviewed in Refs. 5 and 34 and discussed in Ref. 26). For example, the assembled pre-BCR might already constitutively signal either from the endoplasmic reticulum (ER) or from the cell surface. Alternatively, signals might only be initiated from the cell surface after interaction of the pre-BCR with a ligand either presented on or deposited by stroma cells in the extracellular matrix.

To start identifying pre-BCR-interacting structures on stroma cells, we have produced soluble Fab-like pre-BCR fragments consisting of the SL chain and a truncated μH chain containing a V and the first C domain of a μH chain (Fdμ chain). We show in this manuscript that Fab-like, soluble pre-BCR fragments bind in a specific and saturable manner to stroma and other adherent cell lines, but not to several B and T lymphoid suspension cell lines. Furthermore, this binding does not seem to rely on the presence of a μH chain, because the SL chain alone is also capable of interacting with a stroma cell line. We conclude from these findings that the pre-BCR interacts, presumably via its SL chain, with a structure found on the surface of stroma cells, and speculate that this structure is part of a pre-BCR ligand.

DNA fragments encoding the mature form of mouse VpreB, λ5, κL chain as well as the V (VH) and first C (Cμ1) domain of the μ chain were amplified by PCR from plasmids containing the corresponding cDNA sequences with the following primers: for VpreB, 5’-TAGGGCTTACCATGAA GGTTCGTGAAAGCTGAGCAGGAAATGTCGATC-3’ and 5’-TCTAG AGAGAGGCTGAATATAGATTTCATCCAGATGC-3’ and 5’-TCTAG AGAGAGGCTGAATATAGATTTCATCCAGATGC-3’ and 5’-TCTAGGCTTACCATGAA GGTTCGTGAAAGCTGAGCAGGAAATGTCGATC-3’ for the μ chain, 5’-TCTAGGCTTACCATGAA GGTTCGTGAAAGCTGAGCAGGAAATGTCGATC-3’ and 5’-TCTAGGCTTACCATGAA GGTTCGTGAAAGCTGAGCAGGAAATGTCGATC-3’ for the κL chain. PCR products were purified, ligated into the pCR2.1 cloning vector (Invitrogen, Groningen, The Netherlands), and verified by DNA sequencing using the Big-Dye sequencing kit from PerkinElmer Biosystems (Warrington, U.K.). A *Smal*/XhoI λL chain fragment was isolated from the respective pCR2.1 vector and ligated into the transfer vector pFASTDual (Life Technologies). An Fdμ fragment was isolated as a *BamH*I/XhoI fragment and ligated into both the transfer vector pFASTBac1 (Life Technologies) and pFASTDual-κL vector. A *Sal*I/Adv fragment was ligated from the pCR2.1 cloning vector into the pCR2.1 cloning vector (Invitrogen, Groningen, The Netherlands), and verified by DNA sequencing using the Big-Dye sequencing kit from PerkinElmer Biosystems (Warrington, U.K.). A *Sal*I/Adv κL chain fragment was isolated from the respective pCR2.1 vector and ligated into the transfer vector pFASTDual (Life Technologies). An Fdμ fragment was isolated as a *BamH*I/XhoI fragment and ligated into both the transfer vector pFASTBac1 (Life Technologies) and pFASTDual-κL vector. A *Sal*I/Adv fragment was ligated from the pCR2.1 cloning vector into the pFASTDual vector. The transfer vector pFASTDual-VpreB/A5 was constructed by inserting a *Sal*I fragment from the respective pCR2.1 vector into the pFASTDual-VpreB transfer vector. To assemble a vector encoding VpreB, A5, and Fdμ (pFAST-VpreB/A5/Fdμ), a *Sal*I/Aval fragment containing the SV40 poly(A) site and the melittin-Fdμ sequence under the control of the polyhedrin promoter was isolated from pFASTBac1-Fdμ, blunt ended with Klenow enzyme, and ligated into the *Sal*I site of the pFASTDual-VpreB/A5 transfer vector.
Production of recombinant proteins in insect cells

Recombinant virus was produced by first transforming competent DH10BAC Escherichia coli cells with recombinant pFAST transfer vectors, as described in the instruction manual from Life Technologies for the BAC-TO-BAC Baculovirus Expression System. Recombinant virus was produced by transfecting SF21 cells with recombinant baculovirus DNA isolated from recombinant DH10BAC E. coli clones. Virus titer in the growth medium was determined by the end point dilution. Monolayer cultures of SF21 cells from S. frugiperda were maintained at 27°C in TC100 medium supplemented with 10% heat-inactivated FCS (Life Technologies) and infected with recombinant virus with a multiplicity of infection of 5–10. Cells and cell culture supernatants were harvested 3 days after infection. Cell pellets were solubilized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 14 μg/ml aprotinin, 1 mM PMSF, 1 μg/ml pepstatin, and 2 μg/ml leupeptin, and spun for 10 min at 13,000 rpm in a microfuge. Cellular lysates were collected at 13,000 × g for 10 min, and supernatants (lysates) were collected. Cell culture supernatants were supplemented with 1 mM PMSF protease inhibitor.

Metabolic labeling, immunoprecipitation, gel electrophoresis, and Western blotting

Metabolic labeling was performed as described (47). Briefly, 107 ST2 cells in 15 ml of methionine-free labeling medium were incubated overnight with 50 μCi/ml 35S TransLabel (ICN Biomedicals, Eschwege, Germany). Cells were lysed for 30 min on ice in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 14 μg/ml aprotinin, 1 mM PMSF, 1 μg/ml pepstatin, and 2 μg/ml leupeptin, and spun for 10 min at 13,000 rpm in a microfuge. Cellular lysates were incubated with 1 ml of cell medium from uninfected, Fab virus-infected, or pre-BCR virus-infected insect cells for 3 h on ice. Immuno complexes were precipitated with either the FS1 mAb (anti-α5) or goat anti-κ Abs for 2 h on ice, followed by protein G-Sepharose (Pierce, Rockford, IL), separated on a 10% SDS-Laemmli polyacrylamide gel, and detected by fluorography.

For Western blotting, proteins were precipitated from 1000 μl of cell culture supernatant of uninfected or infected insect cells with the respective primary Ab for 2 h on ice, followed by incubation with immobilized protein G-Sepharose beads. Proteins in cell lysates, cell culture supernatants, and immunoprecipitates were separated on 12% SDS-Laemmli polyacrylamide gels and analyzed by Western blot analysis, as previously described (47). Briefly, separated proteins were transferred onto Protean nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% nonfat Carnation dry milk (Nestle, Glendale, CA) in TBS, incubated first with appropriate unconjugated primary Abs, followed by secondary HRP-conjugated Abs, and developed with the ECL method (Amersham, Freiburg, Germany).

Flow cytometry

Adherent cells were first detached with accutase (PAA Laboratories, Colbe, Germany) at 37°C for 15 min and washed once in ice-cold PBS. Cells (5 × 105) were membrane stained for 2 h on ice with 100 μl of supernatant of uninfected or infected insect cells. Cells were then washed and incubated with either unconjugated primary or fluorochrome-conjugated Abs for 30 min on ice. Unconjugated Abs were detected with appropriate fluorochrome-conjugated secondary Abs. Finally, cells were washed and fluorescence was determined by flow cytometric analysis with a FACScanCalibur (BD Biosciences, Mountain View, CA). Flow diagrams were obtained by analyzing the primary data with the CellQuest software program (The Scripps Research Institute, San Diego, CA). After each staining, cells were washed three times with ice-cold PBS (PBS supplemented for flow cytometry with 0.1% NaN3 and 1% BSA).

Results

The major goal of this study was to determine whether the pre-BCR binds specifically to stroma-derived cell lines and, if so, which component of the pre-BCR is required for this interaction. To address these questions, we first inserted DNA fragments encoding the SL chain components VpreB and λ5 as well as a Fdμ chain (consists of a V H and the first C domain (Cμ1) of the μ gene) into the same baculovirus-based transfer vector. We then expressed the genes in insect cells, produced soluble pre-BCR molecules, and determined by flow cytometry whether the soluble pre-BCR interacts with lymphoid and stroma-derived cell lines.

Production of recombinant Fab-like soluble pre-BCR and BCR fragments

Several attempts to produce soluble pre-BCR molecules in insect cells by infecting them stepwise or simultaneously with recombinant VpreB, λ5, and Fdμ viruses failed. Therefore, we inserted expression cassettes for the mature forms of VpreB, λ5, and Fdμ into the same baculovirus-based transfer vector between two bacterial transposon elements (Fig. 2A). Similarly, we constructed recombinant baculovirus DNA encoding a Fab-like BCR fragment by inserting expression cassettes for a conventional κL chain and a Fdμ chain into the same baculovirus transfer vector (Fig. 2B).

To increase the secretion of heterologous proteins in insect cells (46), we replaced the mouse leader sequences of all four chains with the 21-aa-long honeybee melittin leader (Fig. 2C). Recombinant viruses were produced, amplified, and used to infect SF21 insect cells, as described in Materials and Methods. Three bands with expected molecular masses of 30 kDa for Fdμ, 21 kDa for λ5, and 16 kDa for VpreB could be detected under reducing conditions by immunoblotting with Abs reactive against (His)6 (detects recombinant Fdμ chains), VpreB, and λ5, respectively, in cellular lysates and culture media of infected cells (Fig. 3Aa), but not of uninfected cells (data not shown). When we infected insect cells with virus encoding only one of the pre-BCR constituents, we detected with the corresponding Abs signals for Fdμ, λ5, and VpreB on immunoblots in cellular lysates, but not in culture media (data not shown), indicating that none of the pre-BCR components, when synthesized alone, can correctly fold and pass the stringent ER quality control.

FIGURE 2. Generation of baculovirus-based expression vectors. A, The baculovirus-based expression vector pFAST-VpreB/λ5/Fdμ contains the backbone of the pFASTDual vector and sequences encoding the mature forms of VpreB, λ5, and Fdμ, each of which is fused to the leader sequence from honeybee melittin (open rectangles). The inserted sequences are either under the control of baculovirus polyhedrin (open arrow) or the p10 (filled arrows) promoters. The poly(A) addition sites are presented as gray-filled arrows, and the transposons from the Tn5 transposon as gray triangles. ApR, ampicillinase gene. B, pFAST-κL/Fdμ is identical to the vector shown in A, except that the VpreB and λ5 expression cassettes were replaced by a sequence containing the poly(A) signal of SV40 and the coding region of the melittin leader fused to the mature form of a κL chain under the control of the p10 promoter. C, Partial N-terminal amino acid sequences of recombinant λ5, VpreB, Fdμ, and κL chain, each of which contains the 21-aa-long leader peptide of honeybee melittin (indicated by a thick line above the sequence).
We also identified on immunoblots under reducing conditions κL (~27 kDa) and Fdμ chains (~30 kDa) in cellular lysates and culture medium from cells infected with recombinant κL/Fdμ virus (Fig. 3ab), but not from uninfected cells (data not shown). However, in contrast to VpreB or λ5, κL chains are secreted in the absence of Fdμ production (data not shown). However, this result was not surprising, because many L chains are secreted by plasma cell lines in the absence of IgH chains (reviewed in Ref. 48).

To determine whether VpreB, λ5, and Fdμ form trimeric complexes, we incubated the culture medium with a mAb that recognizes only the assembled pre-BCR (clone SL156), but not free SL chain (44), separated the immunoprecipitated proteins under reducing conditions, and analyzed the composition of the immunoprecipitates with Abs against each individual chain by immunoblotting. VpreB, λ5, and Fdμ could be detected with the corresponding Abs on immunoblots in electrophoretically separated anti-pre-BCR precipitates from culture medium of cells infected with VpreB/λ5/Fdμ virus, but not from medium of uninfected cells (Fig. 3Ba). Similar results were obtained when we analyzed BCR fragments immunoprecipitated from culture medium of cells infected with κL/Fdμ virus under nonreducing conditions, that is, Abs against κL and Fdμ chains coprecipitated Fdμ and κL chains, respectively (Fig. 3Bb).

We also found that SL chains are disulfide linked via λ5 with Fdμ chains, because immunoblot analysis of culture medium from cells infected with VpreB/λ5/Fdμ virus revealed under nonreducing conditions a single band of ~55-kDa that reacted with Abs against μH chain and λ5 (data not shown). Similarly, Abs against μH and κL chain detected an ~55-kDa band in medium from cells infected with κL/Fdμ virus (data not shown).

From these findings, we conclude that insect cells infected with recombinant VpreB/λ5/Fdμ and κL/Fdμ virus produce and secrete trimeric Fab-like pre-BCR and dimeric Fab-like BCR molecules, respectively.

Soluble Fab-like pre-BCR fragments bind to adherent, but not to suspension, cells

If an interaction of a pre-BCR with a stroma-derived ligand is critical for early B cell maturation, one would expect that a soluble pre-BCR molecule binds to stroma lines, but not to B or T lymphoid cell lines. To address this question, we incubated several bone marrow-derived stroma and lymphoid cell lines with culture medium from insect cells infected with VpreB/λ5/Fdμ virus (produce soluble Fab-like pre-BCR fragments). Pre-BCR binding was then analyzed by flow cytometry after staining the cells with rat mAbs against VpreB (clone Vp245), λ5 (clone LM34), or pre-BCR (clone SL156), followed by fluorochrome-conjugated secondary anti-rat Abs. The anti-pre-BCR Ab SL156 reacts only with a pre-BCR consisting of VpreB, λ5, and μH chain, but not with SL chain alone (44) and should, therefore, detect only binding of pre-BCR, but not binding of free SL chain. Culture media from uninfected insect cells and from cells infected with recombinant κL/Fdμ virus (produce soluble Fab-like BCR fragments) served as controls to assess the specificity of the pre-BCR binding.

Flow cytometric analysis revealed binding of Fab-like pre-BCR, but not of Fab-like BCR fragments, to the adherent mouse stroma cell lines ST2 and PA6 and the stroma/fibroblast line NIH3T3 (Fig. 4A). The binding of the pre-BCR was detectable regardless of whether we used Abs against λ5 (Fig. 4A) and VpreB (for ST2, see Fig. 6B) or the anti-pre-BCR Ab SL156 recognizing an epitope of...
the complete pre-BCR (Fig. 4A). In contrast, specific binding of Fab-like BCR fragments could not be detected with any of the lines (Fig. 4A). Based on these findings and the fact that the Ab SL156 detected binding of the pre-BCR, but not of SL chain alone (compare Fig. 6Bc with 6Bf), we conclude that soluble pre-BCR molecules bind specifically to ST2 cells.

We also detected specific binding of soluble pre-BCR, but not of soluble BCR fragments to other adherent cell lines, such as 293T, a human embryonic kidney line; Chinese hamster ovary (CHO), a hamster ovary cancer line; HeLa, a human cervix cancer line; and HEp2, an epidermal larynx carcinoma line (data not shown). In contrast, as shown in Fig. 4B, pre-BCR binding could not be detected on lymphoid suspension lines, such as the immature B cell lines WEHI231 and NYC31, the pre-B cell line TK, the plasmacytoma J558L (data not shown), and the T cell lines Jurkat, EL4, and DO11.10. These findings indicate that soluble pre-BCR fragments bind to adherent lines from various species, but not to lymphoid suspension lines.

One could speculate that suspension cells did not bind soluble pre-BCR fragments, because these cells were, in contrast to adherent lines, not treated with accutase before staining. However, accutase treatment of suspension lines TK, DO11.10, and NYC before staining did not result in pre-BCR binding (Fig. 4B). In addition, pre-BCR binding could still be detected with ST2 cells that were detached from the flask by treatment with EDTA (Fig. 4Cc). Therefore, the accutase treatment is not a prerequisite for pre-BCR/ligand engagement.

In contrast to Abs against VpreB, A5, and pre-BCR, Abs against μH chain or the (His)$_5$ tail at the C-terminal end of the Fd$_\mu$ chain did not detect pre-BCR binding to ST2 and other adherent cell lines (data not shown). We were surprised at this finding, because both anti-μH chain and anti-(His)$_5$ Abs were able to precipitate pre-BCR molecules from cell culture medium (data not shown). One explanation for the failure of anti-μ and anti-(His)$_5$ Abs to detect pre-BCR binding could be that the corresponding anti-μ epitopes in the C region of Fd$_\mu$ are masked in cell-associated pre-BCR.

Several stroma cell-derived polypeptides whose binding to pre-B cells depends on the presence of divalent cations have been identified (49). However, these polypeptides can be excluded as potential ligands for the pre-BCR, because pre-BCR binding could still be detected in the presence of EDTA, a chelator for divalent cations (compare Fig. 4Ca with Fig. 4Cd). The binding of pre-BCR to ST2 cells depends on the presence of a trypsin-sensitive structure, because, in contrast to accutase (50), trypsin treatment abolished the binding of pre-BCR to ST2 cells (Fig. 4Cb). Based on these findings, we conclude that the interaction of pre-BCR and ST2 cells does not require divalent cations and is mediated by a trypsin-sensitive structure on ST2 cells.

One important characteristic of a specific receptor/ligand interaction is that the binding of the ligand with its cognate receptor can be saturated. To determine whether the binding of pre-BCR to ST2 cells is saturable, ST2 cells were incubated with serial dilutions of culture medium from insect cells infected with VpreB/A5/Fd$_\mu$ virus. Pre-BCR binding was detected by flow cytometry with the anti-A5 mAb LM34 (Fig. 5, A and B), and mean fluorescence values were plotted vs the amount of pre-BCR added to ST2 cells (Fig. 5C). The titration analysis yielded a saturation curve typical for a specific receptor/ligand interaction; that is, mean fluorescence values increased at first and then tapered off at a pre-BCR concentration of ~50 ng/ml (Fig. 5C). In contrast, BCR binding could hardly be detected and did not increase significantly by increasing the concentration of soluble BCR fragments (Fig. 5C). We conclude from these findings that the interaction of the Fab-like soluble pre-BCR fragment with ST2 stroma cells is saturable and specific.

**SL chain alone binds to ST2 stroma cells**

Pre-BCR complexes harbor usually different V$_H$ region sequences. Therefore, pre-BCR/ligand interactions should be independent of the utilized V$_H$ region and mediated by an invariant structure of the pre-BCR. If this structure is associated with the invariant SL chain, one would expect that SL chain binds to ST2 cells in the absence of μH chain.

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**FIGURE 4.** Flow cytometric analyses of pre-BCR and BCR binding to various cell types. A, Cells were detached from cell culture flasks with accutase, washed, and incubated for 2 h on ice with 100 μl of cell culture supernatant from uninfected insect cells (shaded histograms) and insect cells infected (filled histograms) with either VpreB/A5/Fd$_\mu$ virus (pre-BCR) or κL/Fd$_\mu$ virus (BCR). Pre-BCR binding was detected by staining the cells with either the monoclonal rat (IgG) Ab LM34 (α-A5) or the SL156 Ab (α-pre-BCR), followed by FITC-conjugated Abs against rat IgG. BCR binding was detected with FITC-conjugated goat Abs against mouse κL chain (α-κL). Cell-associated fluorescence was determined by flow cytometry. B, Suspension cells either pretreated (+ Accutase) or untreated (− Accutase) with accutase were washed and incubated for 2 h on ice with 100 μl of cell culture supernatant from uninfected insect cells (shaded histograms) or insect cells infected (filled histograms) with VpreB/A5/Fd$_\mu$ virus (pre-BCR). Binding of pre-BCR fragments was detected with anti-A5 Abs, as described in A. C, ST2 stroma cells were detached from cell culture flasks with accutase (a and d), trypsin (b), or PBS/EDTA (c) and stained with cell culture supernatants from uninfected insect cells (shaded histograms) or insect cells infected with VpreB/A5/Fd$_\mu$ virus (filled histograms) in the absence (a−c) or presence (d) of 5 mM EDTA. Binding of pre-BCR fragments was detected with anti-A5 Abs, as described in A.
To address this question, we first produced soluble SL chain by infecting Sf21 cells with a recombinant virus encoding the mature forms of VpreB and λ5. Both SL chain components could be detected on immunoblots with the corresponding Abs in lysate and growth medium from insect cells infected with recombinant virus (Fig. 6A), but not in lysate and medium from uninfected cells (data not shown). When we performed a combined immunoprecipitation and immunoblot analysis similar to that described in Fig. 3, we found that VpreB and λ5 form a noncovalently associated dimer in the culture medium of cells infected with recombinant VpreB/λ5 virus (data not shown). Therefore, as a conventional L chain, SL chain correctly folds in insect cells and passes the ER quality control in the absence of a μ chain.

To determine whether SL chain binds to adherent cells, ST2 stroma cells were incubated with culture medium from insect cells infected with VpreB/λ5 virus. Binding of SL chain was detected with rat mAbs against pre-BCR components, followed by fluorescein-conjugated secondary anti-rat Abs. Flow cytometric analysis revealed the binding of SL chain to ST2 cells using rat mAbs against λ5 (Fig. 6Ba) and VpreB (Fig. 6Bb), but not with the rat mAb against the pre-BCR (Fig. 6Bc). In contrast, all three Abs detected binding of pre-BCR fragments to ST2 cells (Fig. 6B, d–f).

**FIGURE 5.** Titration analysis of soluble pre-BCR and BCR binding to ST2 stroma cells. ST2 cells were detached with accutase from cell culture flasks and incubated, as described in Fig. 4A, with serial dilutions of cell culture media (1 ml), starting with 200 ng/ml from insect cells either infected with VpreB/λ5/Fdμ virus (pre-BCR) or κL/Fdμ virus (BCR). Pre-BCR and BCR binding were detected with anti-λ5 (clone LM34) and goat anti-κL chain Abs, respectively, followed by appropriate FITC-conjugated secondary Abs. A. Forward and side scatter analysis. B. Fluorescence intensities (F) of cells within the gates shown in A were determined by FACS. The results of the staining with supernatants containing the highest concentrations of pre-BCR and BCR (200 ng/ml) and supernatants of uninfected cells (0) are shown. C. Mean fluorescence values (F Mean) were calculated from the fluorescence intensities of cells marked in B with a line and plotted vs the concentration of soluble pre-BCR or BCR fragments. Approximate concentrations of soluble pre-BCR and BCR fragments were estimated by comparing anti-His signals of the Fdμ chain with signals of a known amount of a His-tagged standard protein on Western blots (data not shown). The results of one of three independent experiments are presented.

**FIGURE 6.** Flow cytometric analysis of SL chain binding to ST2 cells. A. Proteins in cellular lysates (L) and cell culture supernatant (SN) from cells infected with VpreB/λ5 virus were separated on 12% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Membranes were incubated with the respective primary Abs (indicated below the blots) against λ5 (clone FS1) and VpreB (rabbit antiserum; see Fig. 4), followed by HRP-conjugated secondary Abs, and developed by ECL. The signal with an apparent molecular mass of 60 kDa can also be detected with anti-VpreB serum in culture supernatants from uninfected insect cells (data not shown). B. Cells were detached from culture flasks with accutase, washed, and incubated for 2 h on ice with 100 μl of cell culture supernatant of uninfected insect cells (shaded histograms) or cells infected (filled histograms) with either VpreB/λ5 virus (SL chain) or VpreB/λ5/Fdμ virus (pre-BCR). Binding of SL chain and pre-BCR to ST2 cells was detected by flow cytometry with the indicated rat mAbs against mouse VpreB (Vp245), λ5 (LM34), and pre-BCR (clone SL156), followed by FITC-conjugated anti-rat Abs. The same results were obtained in two other independent experiments.
Based on these findings, we conclude that both the soluble pre-BCR as well as free SL chain interact with ST2 cells, and that this interaction is at least in part mediated by an invariant epitope on the SL chain and a trypsin-sensitive structure on ST2 cells.

**Soluble pre-BCR specifically precipitates a 135-kDa polypeptide from ST2 lysate**

To start identifying a pre-BCR-binding molecule, we performed an immunoprecipitation analysis of metabolically labeled proteins from ST2 cells with cell culture medium from cells infected with the VpreB/A5/Fdμ virus (pre-BCR). Medium from uninfected cells (−) and from cells infected with Fdμ/SL virus (BCR) served as negative controls. Immunocomplexes were then precipitated either with anti-λ5 (clone FS-1) or anti-κ Abs. Electrophoretic analysis of pre-BCR/anti-λ5-precipitated [35S]-labeled proteins from ST2 cells revealed a band with an apparent molecular mass of 135 kDa (Fig. 7, lane 1). However, this band was not detectable in anti-λ5 (Fig. 7, lane 2), BCR/anti-κ (Fig. 7, lane 3), and BCR/anti-κ precipitates (Fig. 7, lane 4). Based on these findings, we conclude that the pre-BCR specifically interacts with a ST2-derived polypeptide with a molecular mass of 135 kDa.

**Discussion**

An early checkpoint, at which B lymphoid cells are screened for the presence of an IgL chain-pairing μH chain, has been identified between the pro-B and pre-B cell stage. Although it is clear that only B lymphoid cells with a functional pre-BCR pass this early checkpoint at a normal rate (reviewed in Refs. 4–8 and 34), it is still unclear how a maturation signal is initiated. The existence of a signal-inducing extracellular pre-BCR ligand, either deposited on or secreted by neighboring stroma cells, has long been discussed, but to date has never been described.

To search for a stroma-derived pre-BCR ligand, we first produced soluble Fab-like pre-BCR fragments consisting of a truncated μH chain (Fdμκ) and a covalently associated bona fide SL chain in insect cells using the baculovirus-based expression system. This approach has successfully been used to produce complete Abs (51) and Fab-like IgG fragments (52), as well as soluble pre-BCR molecules consisting of a Fdμκ and, in contrast to our studies, a single chain VpreB-A5 fusion protein (53). The first major finding of our study is that insect cells infected with a recombinant VpreB/A5/Fdμκ baculovirus secrete a trimeric pre-BCR complex consisting of a Fdμκ and a disulfide-linked bona fide SL chain. This is the first time that a heterologous protein complex consisting of three distinct polypeptide chains has successfully been synthesized in the baculovirus-based insect expression system. The soluble pre-BCR fragments behave biochemically like a pre-BCR produced by pre-B cells. For example, A5 is disulfide linked to μH chain in pre-BCR of pre-B cells (13–15) and in Fab-like pre-BCR fragments secreted by infected insect cells (data not shown). In addition, the mAb SL156, which recognizes an epitope formed by all three pre-BCR chains (44), immunoprecipitated pre-BCR fragments from insect cells infected with a virus encoding Fdμκ, VpreB, and A5 (Fig. 3B). Therefore, we believe that the soluble pre-BCR fragment secreted by insect cells has a structure similar to that of the corresponding part of a pre-BCR produced by pre-B cells.

The second and more important finding is that the soluble pre-BCR interacts in a specific and saturable fashion with adherent cell lines, including the widely used stroma lines PA6 and ST2, but not with several B and T lymphoid suspension cells. This finding shows for the first time that the pre-BCR binds specifically to a structure found on the surface of adherent cell lines, and supports the idea that a pre-BCR ligand might exist on stroma cells. SL chain could participate in this binding, either directly by interacting with a cross-linking ligand, or indirectly by inducing a conformational ligand-interacting epitope in the μH chain. We favor the first possibility, since SL chain alone interacts with ST2 cells in the absence of a μH chain (Fig. 6). However, we cannot completely exclude that certain epitopes, for example in the V region of a μH chain, can influence the interaction of a pre-BCR with stroma cells. Indeed, pre-B cells using Vµ12 μH chains with particular CDR3 regions seem to be trapped at the pro-B cell stage, despite the fact that these μH chains assemble with SL chain and are transported to the cell surface of a transformed pre-B cell (54). One explanation for the inability of some Vµ12 μH chains to foster maturation could be that some Vµ12 regions change the conformation of the SL chain and thus prevent binding of the pre-BCR to its cognate ligand.

If ligand-induced cross-linking of the pre-BCR is a prerequisite to initiate a maturation signal in pre-B cells, pre-BCR complexes should be found on the surface of normal pre-B cells and cross-linking of surface pre-BCR should modulate intracellular signal pathways. In fact, low amounts of surface pre-BCR complexes can be detected on freshly isolated pre-B cells (16, 44, 55), and cross-linking of surface pre-BCR complexes on normal and transformed pre-B cell lines induces signal pathways similar to that observed in mature B cells after cross-linking of the BCR (reviewed in Ref. 34). For example, cross-linking of the pre-BCR on transformed pre-B cell lines with anti-μH chain Abs resulted in intracellular calcium mobilization (56–58), protein tyrosine phosphorylation (59, 60), and translocation of μH chains together with proximal tyrosine kinases and adapter proteins, such as Syk and BLNK, into lipid microdomains (60). The fact that stroma cells are required for pre-BCR-induced proliferation of pre-B cells in an in vitro culture system, in which the expression of a transgenic μH chain gene can
be regulated in freshly isolated pro-B cells by a tetracycline-con- trollable transcription factor (26), further supports the idea that the interaction between a putative stroma cell ligand and a pre-BCR might be a prerequisite to initiate pre-BCR-mediated signals in pre-B cells.

However, three sets of experiments seem to challenge the existence of a pre-BCR ligand. First, transgenic mutated \( \mu \)H chains lacking either the C\(_{\mu} \) domain (61) or the C\(_{\mu} \) or C\(_{\mu} \)\( \delta \) domains (62) still promoted the transition of B lymphoid cells from the pre-B to the pre-B stage in A5-deficient mice, indicating that SL chain is not required for initiating maturation signals. However, the authors acknowledged that SL chain-independent maturation signals could also be triggered by self aggregation of the truncated \( \mu \)H chains, which would present an artificial situation not found during normal B cell maturation (34). In support of this idea, the membrane-anchored viral oncogene v-erbB, a homolog of the epidermal growth factor receptor, is capable of transforming cells, despite the fact that it lacks the extracellular ligand binding site (reviewed in Ref. 63). Second, pre-BCR-positive pre-B cells that originated in an in vitro culture system from normal pro-B cells divide several times in the absence of stroma cells or IL-7, suggesting that a pre-BCR ligand on stroma cells is not required for inducing growth of pre-BCR-positive pre-B cells. However, the authors cannot exclude that a pre-BCR cross-linking molecule, present in either the culture medium or the coat of the culture dish, is responsible for this effect. Third, mAbs against the complete pre-BCR (clone SL156) and the pre-BCR components V\( \mu \)B (clone VP245), A5 (clone LM34), and \( \mu \)H chain did not block the development of pro-B into pre-B cells in a fetal liver organ culture system (64), suggesting that the interaction of a pre-BCR with an extracellular ligand is not critical for this maturation step. However, it is possible that the anti-pre-BCR mAbs used in that study, which are identical to the one used in this work, do not interfere with the binding between the pre-BCR and its cognate ligand. This seems to be the case, since the mAbs VP245 (anti-V\( \mu \)B), LM34 (anti-A5), and SL156 (anti-pre-BCR) still reacted with soluble cell-associated pre-BCR (Figs. 4 and 6). One explanation for why Abs against \( \mu \)H chain did not interfere with pre-B cell maturation in an in vitro stroma cell culture system could be that either some epitopes in the C region of \( \mu \)H chain are not accessible for anti-\( \mu \) Abs in cell-associated pre-BCR, or anti-\( \mu \)H chain Abs do not prevent the interaction between pre-BCR and its corresponding ligand. Our findings that polyclonal goat Abs against the C region of \( \mu \)H chain neither reacted with cell-associated pre-BCR nor blocked the binding of soluble pre-BCR to ST2 cells (data not shown) support both ideas.

A major question not resolved in this study is the identity of the stroma cell-associated structure that interacts with the pre-BCR. The pre-BCR-interacting structure is present on all adherent lines used in these studies, but not on lymphoid suspension cells. One feature of most adherent cell lines is that they produce many proteoglycans as well as molecules involved in the biosynthesis and turnover of the extracellular matrix and cell-cell interactions (65). Proteoglycans are good examples of multifunctional surface molecules that participate in cell-cell and cell-matrix interactions, organize cell-matrix adhesion and signaling, serve as coreceptors for extracellular ligands, enhance protein-protein interactions, regulate the activity of growth factors, or concentrate growth factors on the cell surface (reviewed in Refs. 66 and 67). The ligand could either be a member of the large proteoglycan family or be part of the extracellular matrix; therefore, it might not be surprising that pre-BCR binds to many adherent cell lines. The binding of the pre-BCR to ST2 stroma cells does not require bivalent cations, since the addition of the chelator EDTA did not change the binding behavior of soluble pre-BCR fragments. This finding excludes several stroma-derived surface proteins, such as biglycan, osteonectin, collagen type I, clusterin, and matrix glycoprotein sc1 as potential binding partners, since the binding of these factors to pre-B cells requires bivalent cations (49). However, the structure must be part of a molecule containing a protease-sensitive polypeptide, since treatment of trypsin abolished the binding of the pre-BCR (Fig. 4C), and culture medium from insect cells infected with pre-BCR virus, but not with BCR virus, precipitated a 135-kDa polypeptide from ST2 lysates. To speculate about the nature of the 135-kDa polypeptide is beyond the scope of this work. However, the 135-kDa polypeptide is not identical with a previously identified SL chain-associated nonclassical cadherin of 130 kDa, since the message for this protein could not be detected in ST2, PA6, and NIH3T3 cells (68). Future studies will identify the 135-kDa polypeptide and determine whether the binding site for the pre-BCR harbors carbohydrate or lipid sequences and whether the structure is part of an integral membrane protein or an extracellular matrix component.

In summary, we have identified a novel saturable interaction of a soluble pre-BCR fragment with a trypsin-sensitive structure on the surface of adherent cell lines and described a pre-BCR-interacting 135-kDa polypeptide, which provided the first experimental evidence that a pre-BCR ligand might exist. Furthermore, we have shown that this interaction occurs at least in part via a not yet identified structure associated with SL chain. Candidates for the pre-BCR binding motif could be the unique C- or N-terminal tails of V\( \mu \)B and A5, respectively, since these sequences present the only clear structural difference between a SL chain and a conventional L chain (18–19). Therefore, SL chain not only serves as a folding template (11, 37) to facilitate the assembly and transport of a pre-BCR to a signal-competent compartment, but could also participate directly in the signaling process by interacting with a cross-linking ligand either anchored on the surface of stroma cells or deposited by these cells in the extracellular matrix.

Acknowledgments

We thank Birgit Jehr, Dirk Mielzen, and Manuel Selg for critical reading of the manuscript; Ton Rolink, Fritz Melchers, and Jan Jongstra for providing mAbs against the pre-BCR and pre-BCR components; and Edith Roth for excellent technical assistance.

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