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Interaction of Murine Precursor B Cell Receptor with Stroma Cells Is Controlled by the Unique Tail of α5 and Stroma Cell-Associated Heparan Sulfate

Harald Bradl, Jürgen Wittmann, Doreen Milius, Christian Vettermann, and Hans-Martin Jäck

Efficient clonal expansion of early precursor B (pre-B) cells requires signals delivered by an Ig-like integral membrane complex, the so-called pre-B cell receptor (pre-BCR). A pre-BCR consists of two membrane µH chains, two covalently associated surrogate L chains, and the heterodimeric signaling transducer Igαβ. In contrast to a conventional Ig L chain, the surrogate L chain is a heterodimer composed of the invariant polypeptides VpreB and α5. Although it is still unclear how pre-BCR signals are initiated, two recent findings support a ligand-dependent initiation of pre-BCR signals: 1) a pre-BCR/galectin-1 interaction is required to induce phosphorylation of Igαβ in a human precursor B line, and 2) soluble murine as well as human pre-BCR molecules bind to stroma and other adherent cells. In this study, we show that efficient binding of a soluble murine pre-BCR to stroma cells requires the non-IG-like unique tail of α5. Surprisingly however, a murine pre-BCR, in contrast to its human counterpart, does not interact with galectin-1, as revealed by lactose blocking, RNA interference, and immunoprecipitation assays. Finally, the binding of a murine pre-BCR to stroma cells can be blocked either with heparin or by pretreatment of stroma cells with heparitinase or a sulfation inhibitor. Hence, efficient binding of a murine pre-BCR to stroma cells requires the unique tail of α5 and stroma cell-associated heparan sulfate. These findings not only identified heparan sulfate as potential pre-BCR ligands, but will also facilitate the development of appropriate animal models to determine whether a pre-BCR/heparan sulfate interaction is involved in early B cell maturation. The Journal of Immunology, 2003, 171: 2338–2348.

The generation of mature B lymphocytes from pluripotent hematopoietic stem cells is a complex process characterized by the assembly of Ig genes through DNA recombination (1, 2) and the selection of functional B lymphoid cells at multiple checkpoints (3–9). The Ig recombination process begins in early progenitor B (pro-B)3 cells with the assembly of V, D, and J gene segments into an exon encoding the V region of an Ig H chain. If this rearrangement results in the synthesis of the trans-

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3 Abbreviations used in this paper: pro-B, progenitor B; µH chain, IgH chain; SL, surrogate light; ER, endoplasmic reticulum; pre-B, precursor B; pre-BCR, pre-B cell receptor; HS, heparan sulfate; HSPG, HS proteoglycan; BiP, H chain binding protein; CDR3, complementarity-determining region; siRNA, short interfering RNA.

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Although the specific interaction of a soluble mouse (27) as well as a human pre-BCR (28) with stroma cells via its SL chain component has been experimentally verified, it is not clear what part of the SL chain is required for this interaction. Computer modeling, using as a reference the three-dimensional structure coordinates of a conventional Ig L chain, revealed that VpreB contains one V-like and $\lambda_5$ one C-like Ig fold domain (18, 29, 30). When compared with the number of $\beta$-strands in corresponding Ig domains of a conventional Ig L chain, VpreB lacks a J region sequence that typically forms the 9th and final $\beta$-strand in an Ig V domain; in contrast, $\lambda_5$ contains all seven $\beta$-strands found in a conventional C region and one additional $\beta$-strand (B8). The extra $\beta$-strand in $\lambda_5$ seems to replace the missing 9th $\beta$-strand in VpreB, because it is required for the noncovalent association of $\lambda_5$ with VpreB (31). Both VpreB and $\lambda_5$ carry at their $C$- and N-terminal ends, respectively, unique sequences without similarities to any known protein (so-called unique tails). The unique tails are not essential for the association of VpreB and $\lambda_5$, because VpreB and $\lambda_5$ mutants lacking these sequences are still able to efficiently assemble with each other (31). However, three-dimensional molecular modeling revealed that the unique tails protrude from a pre-BCR at the position where the complementarity-determining region (CDR)3 of a conventional L chain is located (29, 30) (H. Lanig, H. Bradl, and H.-M. Jäck, unpublished observations). Therefore, the unique tails of VpreB or $\lambda_5$ should be able to form an epitope that is accessible for a putative ligand on stroma cells.

Stroma cell-associated pre-BCR ligands other than galectin-1 might exist in the mouse system because homologous deletion of the galectin-1 gene did not impair murine B cell development (28). Further, immunoprecipitations from murine stroma cell lysates with a soluble murine pre-BCR identified not yet characterized polypeptides with molecular masses between 95 and 135 kDa (27). Stroma cell-attached heparan sulfate (HS) chains seem to be good candidates for other structures that could interact with a pre-BCR, because heparin, an HS derivative, induces internalization of a pre-BCR but not of a conventional BCR on B lymphoid cell lines (32) and blocks stroma cell-dependent maturation of B and T cell precursors in an in vitro culture system (33).

To determine whether the binding of a pre-BCR to stroma cell requires the unique tails of VpreB and/or $\lambda_5$ and whether HS chains are involved in this interaction, we first produced modified SL chains and Fab-like pre-BCR molecules in insect cells and determined by flow cytometry the influence of SL chain modifications of heparin and of inhibitors and enzymes, both of which reduce surface levels of HS chains on stroma cells, on pre-BCR binding. We found that efficient interaction of a murine pre-BCR and the SL chain with stroma cells requires the unique tail of $\lambda_5$ and stroma cell-associated HS.

Materials and Methods

Cell lines and culture conditions

The mouse stroma line ST2 (34), the mouse fibroblast cell line NIH3T3 (35), the human cervix carcinoma line HeLa (American Type Culture Collection no. CCL-2), and the human epidermal larynx carcinoma line HEp-2 (American Type Culture Collection no. CCL-23) were all maintained at 37°C with 5% CO$_2$ in RPMI medium supplemented with 50 U/ml penicillin, 5% FCS, 1 mM sodium pyruvate, and 2 mM l-glutamine. *Spodoptera frugiperda* insect cells (IPLB-ST2-1; Ref. 36) were maintained as adherent cells in TC100 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin.

Antiserum and mAbs

Rat monoclonal IgG Abs directed against mouse VpreB (clone VP245; Ref. 37) and $\lambda_5$ (clone LM34; Ref. 37), the monoclonal hamster anti-mouse $\lambda_5$ Ab, FSI (11, 38), and unpurified total anti-VpreB rabbit serum generated against a GST-mouse VpreB-fusion protein were previously described (11). The following commercially available Abs were used in this study: Mouse mAbs against penta-His from Qiagen (Hilden, Germany), against rat B4 integrin from BD PharMingen (Heidelberg, Germany), and against S$\delta$ HS (clone 3G10, recognizes a neo-epitope on HS stumps after cleavage with heparitinase) and against human galectin-1 from Medac (Wedel, Germany); FITC-conjugated goat Abs against IgG from mouse (H+L) and rat (Fcγ-specific), rabbit anti-goat IgG (H+L), goat anti-hamster IgG (H+L), and HRP-conjugated rabbit anti-hamster IgG Abs from Jackson ImmunoResearch Laboratories (Dianova, Hamburg, Germany), and HRP-conjugated goat IgG Abs against rabbit and mouse IgG (H+L) from Bio-Rad (München, Germany).

Construction of baculovirus transfer vectors

DNA fragments encoding modified forms of mouse VpreB or $\lambda_5$ were amplified by PCR from plasmids harboring the corresponding cDNA sequences with the following 5′ and 3′ primers: for VpreBDU, 5′-TACGCTACATGAGGTCTCTAGTGAAACGGTACATTGTATCCCTAGGTGTTATCATAAGCTCATTATATACCGACCGCTGCTGATCCTC′-3′ and 5′-TTCTGACTACCTCGGAGCCACCCAC-3′; for $\lambda_5$, 5′-TCGCCGGGACATGAGCTTATGAGAAACGGTACATTGTATCCCTAGGTGTTATCATAAGCTCATTATATACCGACCGCTGCTGATCCTC′-3′ and 5′-TTCTGACTACCTCGGAGCCACCCAC-3′; and for $\lambda_5$S8, 5′-TCGCCGGGACCATGAGCTTATGAGAAACGGTACATTGTATCCCTAGGTGTTATCATAAGCTCATTATATACCGACCGCTGCTGATCCTC′-3′ and 5′-TTCCCCGGGACATGAGCTTATGAGAAACGGTACATTGTATCCCTAGGTGTTATCATAAGCTCATTATATACCGACCGCTGCTGATCCTC′-3′. All PCR primers contained specific restriction sites (underlined) to allow cloning into appropriate vectors. The sequence encoding the honeybee mellitin leader (27) was engineered into each of the 5′ primers downstream of the restriction site and the sequence for six histidines into the C terminus of the $\lambda_5$S8 3′ primer. PCR products were purified, ligated into the pCR2.1 cloning vector (Invitrogen, Groningen, NL), and verified by DNA sequencing using the Big-Dye sequencing kit from Perkin Elmer Applied Biosystems (Warrington, U.K.). SmallXhol $\lambda_5$ fragments were isolated from the respective pCR2.1 vector and ligated into the transfer vector pFASTDual (Life Technologies). BamH1/Xhol VpreB fragments were isolated from the pCR2.1 vector and ligated into the pFASTDual vector harboring the $\lambda_5$ fragments.

Production and purification of recombinant proteins

Recombinant proteins were produced in insect cells as previously described (27). For affinity purification of the recombinant soluble pre-BCR, 15 ml of cell culture supernatant of VpreBDU-infected cells were mixed with binding buffer (10 mM sodium phosphate, pH 7.5), passed through a 0.45 μm filter and separated on a HiTrap heparin-Sepharose column (Amersham, Freiburg, Germany) with a flow rate of 1 ml/min using an LP Chromatography System from Bio-Rad. The column was washed with binding buffer (10× column volume) and eluted with 10 mM sodium phosphate, 1.5 M NaCl, pH 7 (5× column volume). Fractions of 1 ml were collected, dialyzed, and analyzed by Western blotting and flow cytometry.

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. A total of 5 × 10$^5$ cells were membrane stained for 2 h on ice with either Primary Abs diluted in 50 μl PBS supplemented for flow cytometry with 0.1% NaN$_3$ and 1% BSA, or 200 μl culture supernatant of uninfected and infected insect cells or 50 μl of affinity purified fractions. Cells were then washed, incubated with fluorochrome-conjugated or unconjugated secondary Abs for 30 min on ice. Unconjugated Abs were detected with appropriate fluorochrome-conjugated tertiary Abs. After each staining, cells were washed with ice-cold PBS supplemented for flow cytometry as previously described, and fluorescence was determined by flow cytometric analysis with a FACScan (BD Biosciences, Mountain View, CA). Flow diagrams were obtained by analyzing the primary data with the CellQuest software program (The Scripps Research Institute, La Jolla, CA).

To determine whether HS side chains are involved in pre-BCR binding, ST2 cells were cultivated for 20 h in RPMI medium in the absence or presence of the sulfation inhibitor sodium chloride (50 mM); alternatively, detached cells were digested for 1 h at 37°C with 1 μl/mg heparitinase (Medac). For inhibition experiments with lactose, ST2 stroma and HeLa cells were cultured as described (28) for 2 h at 37°C in RPMI medium supplemented with 0.5 M lactose or maltose.
Stable knockdown of galectin-1 through RNA interference with short interfering RNA (siRNA)

A 19-molong DNA sequence downstream of an “AA” motif and with a guanine cytosine content of ~50% was selected within the coding sequence of human galectin-1. A BLAST search confirmed that the selected sequence 5′-AGACAGCACAACCTGTCG-3′ (corresponding to nucleotide residue 111–129 in the human cDNA clone with GenBank accession number NM_002305) is not present in another transcript in the human GenBank database. To generate an expression vector encoding galectin-1 specific siRNA, the selected galectin-1 DNA sequence was inserted into the pSUPER vector as described by Brummelkamp et al. (39). The resulting pSUPER–galectin-1 vector (12 μg) was stably cotransfected with 2.5 μg of the plasmid pTRE2pur harboring a porcymycin N-acetyl-transferase gene (Clontech Laboratories, Heidelberg, Germany) into 106 HeLa cells using Superfect transfection reagent (Qiagen). After 48 h, cells were split 1:10 in fresh RPMI medium. Stable clones were selected with 0.5 μg/ml puromycin (Sigma-Aldrich, Taufkirchen, Germany) over a period of 3 wk, picked, expanded, and analyzed for galectin-1 levels by Western blot analysis.

**Immunoprecipitation, gel electrophoresis, and Western blotting**

Proteins were precipitated from 1 ml cell culture supernatant from uninfected or infected insect cells with the respective primary Ab for 2 h on ice, followed by incubation with appropriate secondary Abs and immobilized protein G-Sepharose beads. Alternatively, proteins from HeLa lysates (5 × 106 cell equivalents) were immunoprecipitated with 2.5 ml cell culture supernatants from uninfected and SL virus-infected insect cells, followed by the rat mAb LM34 (anti-α5) and protein G-Sepharose. Immunoprecipitates as well as affinity-purified proteins were separated on 12% SDS-polyacrylamide gels and analyzed by Western blot analysis as previously described (27). Briefly, separated proteins were transferred onto Protean nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Membranes were blocked with 5% Carnation nonfat dry milk (Nestlé USA, Glendale, CA) in TBS, incubated first with appropriate unconjugated primary Abs, followed by secondary HRP-conjugated Abs and developed with the ECL method (Amersham).

** Primer sequence analysis and secondary structure predictions**

Protein sequences were aligned online (http://www.ch.embnet.org/software/LALIGN_form.html) with William Pearson’s LALIGN program implementing the algorithm of Huang and Miller. Secondary structure predictions were performed online at The Predict Protein Server of the European Molecular Biology Laboratory in Heidelberg (http://www.emb-heidelberg.de/predictprotein).

**Results**

Several recent findings support the existence of a pre-BCR ligand. First, soluble murine (27) and human (28) pre-BCR molecules interact via their SL chain specifically with several adherent lines; second, the interaction of a human pre-BCR is mediated by galectin-1, a surface-exposed galactose-binding protein; and third, incubation of pre-BCR-positive, murine pre-B lines with heparin results in the down-regulation of surface μH chains (32), suggesting that HS chains could be potential binding sites for a pre-BCR. In this report we address the following obvious questions that arose from these findings: First, what part of the SL chain is critical for the interaction of a pre-BCR with adherent cell lines? Second, does a murine pre-BCR in analogy to its human counterpart interact with galectin-1? And finally, are HS structures involved in the binding of a murine pre-BCR to adherent cell lines? To answer these questions, we used flow cytometry to analyze the effects of VpreB and α5 modifications, various HS-metabolizing enzymes and inhibitors and siRNA-induced knockdown of galectin-1 on the binding of the SL chain as well as soluble Fab-like pre-BCR molecule to murine ST2 stroma and human HeLa cells.

**The Ig-like α5 constant region is dispensable for SL chain binding to stroma cells**

To determine whether the Ig-like constant domain of α5 is critical for binding of the SL chain to ST2 stroma cells, we first constructed a baculovirus expression vector harboring the coding sequence for both wild type VpreB chain and a truncated α5 chain consisting of the N-terminal unique tail and the extra α8 strand (Fig. 1A). The α8 strand of α5 was included, because it is required for the correct heterodimerization of VpreB and α5 (31). In addition, six histidine residues (His6 tag) were added to the C-terminal end of the truncated α5 chain to facilitate its detection and affinity purification of the modified SL chain on Ni-NTA columns. Wild type and the modified SL chain were produced in insect cells by infecting them with the appropriate recombinant baculovirus as previously described (27), and cell culture supernatants were analyzed for the presence and association of the SL chain components VpreB and α5U8 by a combined immunoprecipitation/Western blotting assay (Fig. 1B). Monoclonal anti-VpreB (clone VP245; Fig. 1B, lane 3), as well as anti-His Abs (Fig. 1B, lane 4) precipitated an anti-VpreB reactive band with a molecular mass of ~16 kDa and an anti-α5-reactive band with a molecular mass of ~8 kDa from cell culture supernatant of VpreB/α5U8 virus-infected insect cells. Because both molecular masses correspond to that of the theoretically calculated mass of VpreB and α5U8, respectively, we conclude that both VpreB and the truncated α5 polypeptide are produced and released by insect cells in the culture medium as noncovalently associated heterodimers.

Next, we analyzed the interaction of a wild type and truncated SL chain to ST2 stroma cells with a flow cytometric binding assay (27). Because this assay will be used throughout the entire study, the three-step staining procedure should be explained in more detail at this point. First, murine ST2 stroma or human HeLa cervical cancer cells were incubated with cell culture supernatant from insect cells that were infected with baculovirus encoding modified SL chain or pre-BCR molecules (primary staining). Supernatants from cells infected with virus encoding wild type SL chain (VpreB/α5 virus) or Fab-like pre-BCR molecules served as positive controls, and supernatants from uninfected insect cells as negative controls. Cells were then incubated with mAbs (secondary staining) directed against either VpreB (clone Vp245; Ref. 37), α5 (clone LM34; Ref. 37) or the His6 tag, followed by the appropriate FITC-conjugated tertiary Ab (tertiary staining); cell-associated fluorescence was finally detected by flow cytometry.

Using this assay, similar staining intensities for wild type (VpreB/α5) and the truncated SL chain (VpreB/α5U8) were detected on ST2 cells with anti-VpreB Abs (compare in Fig. 1C, a with c). In addition, binding of the His6-tagged truncated SL chain could also be observed with monoclonal anti-His Abs (Fig. 1Cd), indicating that the His6 epitope is accessible for Ab binding and seems, therefore, not to be involved in the binding of VpreB/α5U8 to ST2 stroma cells. If the binding of VpreB/α5U8 to ST2 cells occurs rather via SL chain sequences than via the His6 tag, masking the His6 tag with anti-His Abs should not interfere with the binding of the truncated SL chain. Affinity chromatography on nickel beads revealed that the mouse anti-His Ab (anti-His) that was used to detect cell-bound His6-tagged SL chains in Fig. 1C was indeed able to mask the His6 tag in VpreB/α5U8, because the binding of His-tagged VpreB/α5U8 to nickel beads could be completely blocked by incubating supernatants from VpreB/α5U8-infected insect cells with anti-His Abs before purification (data not shown). Therefore, we repeated the staining of ST2 cells with VpreB/α5U8 supernatants, in which the His tag of VpreB/α5U8 had been masked by anti-His Abs before staining ST2 cells. As seen in Fig. 1D, the intensity of VpreB/α5U8 binding detected with Abs against VpreB was the same, regardless of whether anti-His Abs were absent or present during the primary binding reaction of VpreB/α5U8. Furthermore, the same binding intensities of VpreB/α5U8 preincubated with mouse anti-His Abs could be detected with tertiary fluorochrome-conjugated anti-
mouse Abs, regardless whether mouse anti-His Abs were added or not after binding of VpreB/ASU58 (compare Fig. 1D, d with e). These results indicate that 1) anti-His Abs from the preincubation reaction remained associated with cell-bound VpreB/ASU58, and 2) all His6 epitopes were blocked by anti-His Abs. Hence, the binding of SL chain to ST2 cells is mediated by an epitope associated with VpreB/ASU58, but neither by the C-terminal His tag of the truncated A5 chain nor an epitope in the Ig-like C domain of A5.

The unique tail of A5 is critical for binding of SL chain to ST2 stroma cells

Secondary structure predictions revealed that the so-called unique tails at the N- and C-terminal ends of A5 and VpreB, respectively, constitute the major difference between the SL chain and a conventional Ig L chain (18, 29, 30). In addition, three-dimensional molecular modeling disclosed that the unique tails protrude from a pre-BCR molecule at the same position where the CDR3 of a conventional L chain is located (H. Lanig, H. Bradl, and H.-M. Jäck, unpublished observations). Therefore, we predict that the unique tails of VpreB and A5 should be able to form either alone or together a structure that is accessible for a putative stroma cell ligand.

To test this hypothesis, we constructed baculovirus transfer vectors encoding SL chains that lack the unique tails of VpreB and/or A5. Wild type and modified SL chains, as well as Fab-like pre-BCR molecules, were produced in insect cells and detected by a combined immunoprecipitation/Western blot analysis. Because in this study the FS-1 Ab, the only available Western blot-reactive anti-A5 Ab, binds to an epitope in the unique tail of A5 (data not shown), the production of a tailless A5 chain and its association with VpreB was detected indirectly with the following approach. SL chains and pre-BCR molecules were immunoprecipitated from culture supernatants of infected insect cells with the LM34 Ab, a rat mAb recognizing an epitope in the C-like domain of nonnatural A5. Immunoprecipitated proteins were electrophoretically separated under reducing and denaturing conditions, transferred to a membrane, and analyzed for the presence of VpreB with a rabbit serum recognizing VpreB independent of the presence of the unique tail. When cell culture supernatants from insect cells infected with various recombinant virus were analyzed with this approach (Fig. 2B), we detected wild type VpreB with an expected molecular mass of 16 kDa in anti-A5 precipitates from culture supernatants of VpreB/A5 (Fig. 2B, lane 1) and VpreB/A5ΔU virus-infected cells (Fig. 2B, lane 3); similarly, LM34 anti-A5 Abs coprecipitated VpreBΔU with a predicted molecular mass of 12 kDa from culture supernatants of VpreBΔU/A5 and VpreBΔU/ A5ΔU virus-infected cells. Therefore, we conclude that insect cells infected with recombinant VpreBΔU/A5-, VpreBΔU/A5ΔU, or VpreBΔU/A5ΔU virus produce and secrete dimeric modified SL chains lacking the unique tail of VpreB, A5, or both.

Flow cytometric assays identical with the one described in Fig. 1 revealed that the unique tail of A5, in contrast to that of VpreB, is critical to facilitate efficient binding of the SL chain to ST2 stroma cells, because a clear decrease in fluorescence intensity was only detected when the recombinant SL chain lacked the unique tail of A5 (Fig. 2C, c and d). The same result was obtained, when we examined the binding behavior of a Fab-like pre-BCR lacking the unique tail of A5 (data not shown). Based on these findings, we conclude that the unique tail of A5 is required for efficient binding of a pre-BCR to its respective binding site on ST2 stroma cells.
Galectin-1 does not interact with a murine pre-BCR

The following lines of evidence support the idea that the galactose-binding polypeptide galectin-1 could be one of the long-sought ligands of the human pre-BCR (28). First, the binding of a soluble human pre-BCR and SL chain to murine M55.1 stroma cells can be completely blocked by precultivating the cells in the presence of lactose, a galactose-containing disaccharide. Second, purified human pre-BCR interacted specifically with purified galectin-1 in a BIAcore analysis (Uppsala, Sweden).

However, when we examined the interaction of a murine pre-BCR with galectin-1 in three independent approaches, we found a completely different picture. In contrast to its human counterpart, the murine pre-BCR did not interact via galectin-1 with murine ST2 stroma or human HeLa cells. For example, preculturing murine ST2 stroma (Fig. 3A) as well as human HeLa cells (data not shown), with lactose did not reduce the binding of a soluble murine pre-BCR (Fig. 3Ac) when compared with stainings of cells precultured in medium alone (Fig. 3Ac) or in medium supplemented with the control disaccharide maltose (Fig. 3Ab). Second, Western blot analysis with anti-galectin-1 Abs did not detect galectin-1 in an electrophoretically separated SL chain precipitate from lysates of HeLa cells (Fig. 3B, lane 2), despite the fact that galectin-1 is present in the lysate of the HeLa line used in our studies (Fig. 3B, lane 3). Third and most convincingly, stable knockdown of galectin-1 in HeLa cells through RNA interference using a galectin-1 specific siRNA (40) did not reduce the binding of a soluble SL chain (Fig. 3C). In addition identical saturation curves were obtained regardless of whether clones with high or low levels of...
galectin-1 were analyzed (data not shown). HeLa clones with decreased levels of galectin-1 (galectin-1 knockdown clones) were generated by stable transfection with a pSUPER vector encoding RNA that folds into a double-stranded galectin-1-specific siRNA after transcription (39). As summarized in Fig. 3C, only small amounts of galectin-1 could be detected on Western blots in clones 15 and 33; however, flow cytometry revealed identical SL chain staining intensities regardless of whether we analyzed the knockdown clones 15 and 33, clones with high levels of galectin-1 (clones 19 and 22; Fig. 3C), or wild type HeLa cells (data not shown). Hence, we conclude that in contrast to its human counterpart, the murine SL chain does not require galectin-1 for cell binding to human HeLa and murine ST2 stroma cells.

**Heparin interferes with the binding of a soluble pre-BCR to ST2 stroma cells**

The findings previously reported suggest that a murine pre-BCR uses a binding partner on ST2 stroma cells that is different from galectin-1. Potential candidates could be proteins with a molecular mass between 95 and 135 kDa, because we recently reported that a murine pre-BCR, but not a BCR, precipitates a metabolically labeled protein band in this molecular mass range (27). Several attempts to identify this protein band via mass spectroscopic methods failed or gave nonreproducible results. Therefore, we decided to first narrow the putative ligand to a group of molecules with shared biochemical features, which might allow the enrichment of a putative ligand by conventional chromatographic methods and its identification by a mass spectrometric or protein sequencing approach.

The pre-BCR ligand on stroma cells could belong to a family of proteins with covalently attached HS side chains, because heparin, a HS derivative, induces the internalization of surface µ chains in pre-B cells but not on B cell lines (32). In addition, heparin negatively influences the development of B lymphocyte precursors in an in vitro culture system (33). Both of these effects could be the consequence of an interaction between heparin and a pre-BCR. If this is true, we would predict that heparin blocks or at least reduces the binding of a pre-BCR to ST2 stroma cells, and in fact, this is what we found. When we stained ST2 cells with culture medium from pre-BCR virus-infected insect cells that were preincubated with heparin. A HS derivative, induces the internalization of surface µ chains in pre-B cells but not on B cell lines (32). In addition, heparin negatively influences the development of B lymphocyte precursors in an in vitro culture system (33). Both of these effects could be the consequence of an interaction between heparin and a pre-BCR. If this is true, we would predict that heparin blocks or at least reduces the binding of a pre-BCR to ST2 stroma cells, and in fact, this is what we found. When we stained ST2 cells with culture medium from pre-BCR virus-infected insect cells that were preincubated with heparin, we found that the binding of a pre-BCR to ST2 stroma cells was reduced when heparin was present during the binding reaction (compare results in Fig. 4Aa, a with b). A titration analyses of pre-BCR binding in the presence of increasing amounts of heparin yielded a saturation curve characteristic for a specific receptor/inhibitor interaction (Fig. 4Ac); that is, mean fluorescence values decreased with increasing amounts of heparin and stagnated at a heparin concentration of ~50 ng/ml (Fig. 4Ac).

Because heparin was present during the binding of a pre-BCR to ST2 cells, the previous experiment did not clarify whether heparin interacts with the soluble pre-BCR or with a pre-BCR binding structure on ST2 cells. The latter can be excluded, because preincubation of ST2 cells with heparin did not affect pre-BCR binding (Fig. 4Ad and e). In addition, we applied cell culture supernatant from pre-BCR virus-infected insect cells onto a HiTrap heparin-Sepharose column. The column was washed, and bound material was eluted with a high-salt buffer. Flow through, wash fraction, and eluates were collected and analyzed for the presence of the pre-BCR and its bindings capability to ST2 cells. Western blot analysis of each individual fraction with Abs against the pre-BCR components VpreB, A5, and Fdμ revealed under reducing conditions the presence of all three chains in the first two elution fractions (E1 and E2 in Fig. 4Ba), but not in flow through and wash fractions, indicating that the soluble pre-BCR interacts with immobilized heparin. In addition, flow cytometric analysis revealed pre-BCR binding activity to ST2 cells in elution fraction 1 (E1 in Fig. 4Bc), but as expected from the Western blot results, not in the flow through or in later elution fractions (e.g., E4 in Fig. 4B).

**Binding of a murine pre-BCR to stroma cells requires cell-associated HS**

Because a pre-BCR interacts with heparin, a HS derivative, one could speculate that the interaction of a pre-BCR with ST2 cells is
actually mediated by a stroma cell-associated HS motif. To determine whether surface-exposed HS side chains are critical for a pre-BCR/stroma cell interaction, we first treated ST2 cells with heparitinase, an enzyme that specifically cleaves HS side chains from the cell surface (41), and determined the effect of this treatment on the pre-BCR/ST2 stroma cells interaction. Indirect flow cytometry revealed that when compared with untreated ST2 cells (Fig. 5Aa), heparitinase-treated cells clearly bound much less pre-BCR (Fig. 5Ab). The effect on pre-BCR binding was the result of a specific removal of HS side chains, because the heparitinase treatment generated an neo-epitope on HS side chains (33) that could be detected with the specific mAb 3G10 (anti-neohep in Fig. 5A, c and d) only on enzyme-treated (Fig. 5Ad) but not on untreated ST2 cells (Fig. 5Ac). In addition, unspecific digestion of surface molecules can be excluded, because the treatment did not change the surface level of β1 integrin (Fig. 5A, e and f), a subunit of a cell adhesion molecule (42).

Heparin and HS chains are modified by cellular sulfation (43). If sulfate groups of HS side chains on ST2 cells are critical for a pre-BCR/stroma cell interaction, we expect that inhibition of cellular sulfation will reduce the binding of pre-BCR to ST2 stroma cells. In fact, indirect flow cytometry revealed that ST2 cells that were precultured in the presence of sodium chloride bound much less pre-BCR than ST2 cells that were incubated in medium lacking the inhibitor (compare Fig. 5B, a with b). This was not due to a loss of membrane integrity or unspecific down-regulation of surface proteins, because a mAb against β1 integrin stained equally well regardless of whether ST2 cells were cultured in the presence or absence of sodium chloride.

In summary, efficient interaction of a murine pre-BCR with stroma cells requires the unique tail of A5 and a stroma cell-associated HS chain.

Discussion

The importance of a complete pre-BCR in early B cell maturation has been demonstrated in several transgenic and gene-targeted mouse models (reviewed in Refs. 3–6). It is also accepted that the pre-BCR fulfills most of its functions by inducing intracellular signal pathways and controlling the expression of target genes via tyrosine phosphorylation of the signal transducer Igαβ and downstream kinases (reviewed in Refs. 5, 6, 44–47). In contrast, mechanisms of pre-BCR signal initiation are still controversially discussed in the literature (reviewed in Refs. 5, 6, 48 and discussed in Ref. 27).

One model to explain pre-BCR signal initiation postulates the existence of a pre-BCR ligand either exposed on the surface of neighboring bone marrow stroma cells or deposited by these cells onto the extracellular matrix. Although most of the published findings argue against ligand-induced pre-BCR signaling (reviewed in Refs. 5, 6, 48 and discussed in Ref. 27), several recent findings revived the concept of ligand-induced pre-BCR signaling. First, mouse and human SL chains as well as soluble Fab-like pre-BCR molecules bind specifically to human and murine stroma lines (27, 28). Second, galectin-1, a galectose-specific animal lectin (reviewed in Ref. 49). Third, coculturing of human Nalm6 pre-B cells with murine or human stroma lines induces coclustering of the pre-BCR and galectin-1 (28). Fourth, pre-BCR-induced tyrosine phosphorylation of Igαβ is prevented, when human Nalm6 cells were cocultured with stroma cells, from which membranous noncovalently attached galectin-1 has been removed with the competitive galactose-containing disaccharide, lactose (28). And finally, ST2 stroma cell-induced growth of freshly isolated pro-B cells, in which de novo synthesis of a transgenic μH chain, and consequently the pre-BCR, was controlled by tetracycline, can be observed only in the presence of a complete pre-BCR (21).

The discovery of galectin-1 as a potential pre-BCR ligand seemed to be a big step forward toward our understanding of ligand-induced pre-BCR signaling. However, one caveat of this study was that B cell maturation was not affected in galectin-1 knockout mice (28), implying that other ligands might exist. We have recently identified a specific protein band with a molecular mass in the range of 90–135 kDa in pre-BCR immunoprecipitates of stroma cell lysates (27). So far, however, matrix-assisted laser desorption ionization time of flight mass spectrometry of gel-separated protein bands from pre-BCR precipitates has not yet clearly identified a new pre-BCR interacting protein.

Even without molecular information on a pre-BCR ligand, one could still determine whether a pre-BCR/stroma cell interaction is required for efficient maturation of early pre-B cells. For example, one could first identify the stroma cell-interacting binding epitope in a pre-BCR and then characterize the functionality of a pre-BCR/stroma cell interaction in appropriate cell culture and animal models. Therefore, the first aim of this study was to reveal the pre-BCR region that is required for stroma cell interaction.

Pre-BCR/stroma cell interaction

Identification of the pre-BCR binding epitope. Recombinant SL chains bind specifically to stroma cells (27, 28), indicating that the binding epitope is located on the SL chain components, VpreB and/or A5. Primary and secondary structure predictions as well as three dimensional modeling (18, 29, 30) revealed that the unique...

![FIGURE 5. Effect of reduced HS levels on pre-BCR binding. A. Effect of heparitinase treatment. Detached ST2 stroma cells were first incubated for 1 h at 37°C without (−) or with heparitinase (+), washed, and subsequently stained with (a and b) medium from VpreB/A5/Fdμ virus- (pre-BCR) infected insect cells, (c and d) Abs against a HS neo-epitope (anti-neohep) generated on HSPG by digestion with heparitinase, or (e and f) 1 mAb against β1 integrin (anti-β1). B. Inhibition of cellular sulfation. ST2 cells were first cultivated for 20 h in the absence (−) or presence (+) of sodium chloride, detached from cell culture flasks with accutase, washed and subsequently stained with (a and b) culture medium from VpreB/A5/Fdμ virus- (pre-BCR) infected insect cells or (c and d) 1 Ab against β1 integrin (anti-β1). Pre-BCR and Ab binding were detected in all experiments with appropriate fluorochrome-conjugated secondary reagents as described in Fig. 1C. Cell culture supernatant from uninfected insect cells (light gray histogram) served as negative staining controls.](http://www.jimmunol.org/)

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tails at the C- and N-terminal end of VpreB and A5, respectively, constitute the only clear difference between the SL chain and a conventional Ig L chain. In addition, both tails protrude from SL chains at the position where the CDR3 of a conventional Ig L chain would be located (30) (H. Lanig, H. Bradl, and H.-M. Jäck, unpublished observations). Therefore, both tails should interact with each other and be accessible for stroma cell binding. Indeed, flow cytometric analyses clearly showed that the unique tail of A5, but not that of VpreB, is required for efficient binding of SL chain and a soluble pre-BCR to ST2 stroma cells (Fig. 2C). Hence, the interaction of a murine pre-BCR to ST2 stroma cells is controlled by the unique tail of its A5 chain, a conclusion that is further supported by the finding that Abs against the tail of human A5 prevented the interaction between a human SL chain and galectin-1 in a BIAcore analysis (28).

In addition, primary and secondary structure analyses also predict that the A5 tail might be a good binding partner for a counter structure on the surface of stroma cells. For example, primary sequence analyses revealed an overall acidic structure for murine and human VpreB tails (theoretical pIs of 5.1 and 4.6, respectively) and a basic structure for murine and human A5 tails (theoretical pIs of 12.1 and 12.5, respectively) (Fig. 6 and Ref. 28). Further, secondary structure analysis predicts in the middle of both human and murine A5 tails a short α-helical stretch, which divides these tails in an N- and C-terminal basic region (Fig. 6A). Furthermore, VpreB tails (21 and 24 amino acid residues for murine and human, respectively) are about half the size of murine and human VpreB tails, both of which contain 50 amino acid residues. Considering these structural characteristics and the fact that both tails are in close proximity in a modeled SL chain (30) (H. Lanig, H. Bradl, and H.-M. Jäck, unpublished observations), one could speculate that the entire acidic tail of VpreB is engaged with the C-terminal basic region of the A5 tail. This would leave the N-terminal basic part of A5 upstream of the predicted α-helical stretch free to interact with an acidic structure on the surface of stroma cells.

Besides this new function of the A5 tail, other roles for both VpreB and A5 tails have been discussed. The idea that ionic interactions between the two tails initiate and promote the association of VpreB and A5 chains (28) fell short with the discovery that neither tail was required for VpreB and A5 to assemble into the SL chain (31). The tails might rather be responsible for retaining unassembled SL chains and most of the assembled pre-BCR complexes in an intracellular compartment (50), presumably the ER, through binding to the chaperone H chain binding protein (BiP)

**FIGURE 6.** Comparison of deduced amino acid sequences encoded by the unique tails of mouse and human A5 and VpreB genes. The amino acid translation of the unique tails of murine A5 (A) and murine VpreB (B) are shown on top along with the amino acid comparison of human A5 and VpreB, respectively. Alignments were created by the LALIGN program (see Materials and Methods). Basic residues in A5 are shown in bold. Gaps (−) were introduced to maximize alignments. Amino acid identities are boxed. Regions with a putative α-helical structure are indicated by bars. The positions of the unique tails within A5 and VpreB chains were taken from Ref. 30. The following sequences were used (with GenBank accession numbers in parenthesis): murine A5 (A26166), rat A5 (Z68145), and human A5 (M27749); murine VpreB1 (A28344) and human VpreB1 (S00258).

5 this idea is supported by the finding that “tailless” pre-BCRs are more efficiently transported to the surface of pre-B cell lines than their wild type counterparts (53). Therefore, the tail of A5 might fulfill a dual function in B cell development: on one hand, the A5 tail, either alone or in concert with the VpreB tail, could facilitate the binding of an unassembled SL chain as well as completely assembled pre-BCR complexes to an ER-resident chaperone. This would limit surface expression of a signal-competent pre-BCR and thus, control the proliferative capacity of pre-B cells and consequently preventing leukemogenesis. The finding that increased levels of surface pre-BCR correlates with an increase in proliferation supports this idea (47). In contrast, the basic tail of A5 interacts with an acidic structure exposed on the surface of stroma cells or deposited on the extracellular matrix, thereby concentrating the few pre-BCRs on the surface of a pre-B cell into signal-competent lipid microdomains (44).

**Analysis of murine pre-BCR/galectin-1 interaction.** Recently, galectin-1 has been described by Gauthier et al. (28) as one potential ligand for the human SL chain and pre-BCR. The same authors showed that a pre-BCR/galectin-1 interaction is critical for pre-BCR-induced phosphorylation of Igα in human Nalm6 pre-B cells that were cocultured on stroma cells, implying that a pre-BCR/galectin-1 interaction is important for triggering human pre-BCR signaling. Surprisingly however, we did not detect binding of a murine SL chain and a soluble murine pre-BCR to galectin-1 with assays similar to that used by Gauthier and coworkers (28). For example, treatment of either murine ST2 stroma or human HeLa cells with the competitive galectin-1 ligand, lactose, did not affect murine pre-BCR binding (Fig. 3A). In addition, an immunoprecipitation experiment did not detect galectin-1 in SL chain precipitates from HeLa cell lysates (Fig. 3B). Both results imply that a murine SL chain as well as a murine pre-BCR bind neither to murine nor to human galectin-1. However, galectin-1 removal by lactose could not be monitored because commercial Abs reactive against cell surface-associated galectin-1 by flow cytometry were not available. In addition, slight differences between our immunoprecipitation procedure and the one used by Gauthier and coworkers (28) could change the binding behavior of a murine pre-BCR to human galectin-1 and explain why galectin-1 was absent from murine SL chain precipitates of HeLa lysates. However, analysis of HeLa clones, in which galectin-1 mRNA levels have been reduced by stably transfected galectin-1 siRNA (40), revealed that reduced galectin-1 protein levels affected neither the binding nor the kinetic of murine SL chain binding to HeLa cells (Fig. 3C). Therefore, the binding of a murine pre-BCR to human HeLa cells does not require galectin-1.

Although it is clear from the siRNA inhibition experiment that human galectin-1 is not required for a murine pre-BCR/HeLa cell interaction, we cannot exclude that a murine pre-BCR might still bind to murine galectin-1. This, however, seems unlikely for three reasons. First, lactose washing of murine ST2 cells did not affect pre-BCR/ST2 interaction (Fig. 3A). Second, galectins are highly conserved animal lectins; for example, comparison of protein sequence records for murine (GenBank accession no. P16045) and human (GenBank accession no. P09382) galectin-1 revealed that both sequences are 88.1% identical. Third, neither central B cell maturation nor numbers of peripheral B cells are affected in galectin-1-deficient mice (see Discussion in Ref. 28). Therefore, we conclude that the interaction of a murine pre-BCR with murine ST2 stroma as well as human HeLa cells does not require the presence of galectin-1.

Although we cannot provide a satisfactory explanation for the
observed galectin-1 binding differences between a human and murine pre-BCR, there are some differences between a human and a murine SL chain, which might account for the observed differences. First, human and murine sequences of both the VpreB and A5 tail show only an identity of 56% and 58%, respectively (Fig. 6). Second, the human A5 tail carries two extra basic lysine residues, and human VpreB contains three extra amino acids at its C-terminal end. Third, in contrast to its murine homologue, a human SL chain is not expressed on the surface of freshly isolated pro-B cells (54), indicating that the human but not the mouse A5 tail might be retained by an intracellular protein. In addition, there are also differences between our SL chain preparation and that used by Gauthier et al. (28). For example, all the SL chain constructs used by Gauthier et al. (28) contained His tags at the N- and C-terminal ends of VpreB and A5, respectively. In contrast, we used untagged wild type SL chains for all our binding studies. Depending on the pH micromilieu and the conformational context, a His tag might change the binding specificity of recombinant proteins by favoring unspecific ionic interaction or hydrogen bridges. Depending on the pH micromilieu and the conformational context, a His tag might change the binding specificity of recombinant proteins by favoring unspecific ionic interaction or hydrogen bridges. Depending on the pH micromilieu and the conformational context, a His tag might change the binding specificity of recombinant proteins by favoring unspecific ionic interaction or hydrogen bridges.

Involvement of stroma cell-associated HS. Another new finding of this report is that a pre-BCR as well as the SL chain interact with stroma cell-associated HS chains. This conclusion is based on four findings that are accepted in the field to clearly indicate HS chains as a binding partner for a particular protein. First, heparin blocks the binding of a soluble pre-BCR to ST2 stroma cells (Fig. 4A). Second, a pre-BCR can be purified by affinity chromatography on heparin-Sepharose columns (Fig. 4B). Third, removal of HS chains from ST2 cells with bacterial heparitinase (41) resulted in a reduced binding of a soluble pre-BCR (Fig. 5A). Finally, pre-BCR binding was also reduced when ST2 cells were preincubated with sodium chloride, an inhibitor of cellular sulfation of HS chains (Fig. 5B).

HS are glycosaminoglycans with repeats of disaccharide units consisting of N-acetylglucosamine and hexuronic acid (reviewed in Ref. 55). Because the fine structure of a HS chain can be modified by deacetylation as well as O- and N-sulfation at various positions within a HS chain, the complexity of HS chains exceeds that of DNA (see references in Ref. 56). One or more HS chains are normally covalently attached to a specific core protein and presented as HS proteoglycans (HSPG) on the cell surface. Although the protein part of a HSPG determines localization either to the extracellular matrix, intracellular granules, or the cell surface, the HS chain mediates interactions with extracellular ligands. Through these interactions, HSPGs participate in many events during cell adhesion, migration, proliferation, and differentiation in lymphohematopoiesis (reviewed in Refs. 55, 57). For example, HSPGs are involved in adhesion and long-term maintenance of hematopoietic cells and may influence proliferation and differentiation of various hematopoietic lineages. In addition, stroma cell-associated HSPGs can bind IL-3, GM-CSF, and IL-7, which are presented in a biologically active form to their high affinity receptors on hematopoietic cells (reviewed in Refs. 55, 57). These findings indicate that binding of growth factors by HSPGs might be an important mechanism for regulating hematopoietic differentiation.

Many HS ligands bind via positively charged specific consensus sequences consisting of clusters of lysine, arginine, and glutamine to well-defined sequences on the HS chain (58). In fact, the tail of A5 fulfills all the requirements of a good HS ligand. For example, it has a positive net charge and contains two basic clusters consisting of lysine and glutamine residues (Fig. 6A). Thus, it is tempting to speculate that the interaction of a pre-BCR and ST2 cells is mediated directly via the positively charged N-terminal part of A5 and the HS sequence.

Do we need ligand-induced maturation of early pre-B cells?

Based on published data (Refs. 21, 27, 28 and reviewed in Refs. 3–7) and the findings reported in this report, we would like to present a model in which ligand-induced pre-BCR signaling is only required for a small subset of pre-B cells, i.e., those with very low levels of surface pre-BCRs.

The central molecule in most of the models of pre-BCR signal initiation (overview in Ref. 6) is the SL chain. It is assembled from VpreB and A5 in early pre-B cells, even before the onset of V(D)J rearrangement, and presented in association with B cell-specific galectin-1, a 130-kDa glycoprotein, on the surface of pro-B cells as the so-called pro-BCR (59). Because the SL chain binds specifically to ST2 stroma cells in the absence of a μ chain (27, 28 and Fig. 1C), the pro-BCR could also interact via its SL chain with stroma cells. However, the pro-BCR does not associate with the signal transducer Igαβ (59), and therefore, does not induce pre-BCR-associated intracellular signals.

Once a μ chain is synthesized in early pre-B cells, it assembles even in the absence of a complete SL chain with the Igαβ signal transducer in the endoplasmic reticulum (ER) (Ref. 60 and D. Mielenz and H.-M. Jäck, unpublished observations). Therefore, intracellular pre-BCR-like signals could already be initiated from this intracellular compartment. However, this seems to be unlikely, because in contrast to surface a μH/Igαβ/Igαβ complex (61), an ER-retained μH chain/Igαβ/Igαβ complex cannot be tyrosine phosphorylated in a pervandate model system (D. Mielenz and H.-M. Jäck, unpublished observations). In addition, a particular μ chain that is incapable to pair with A5 and is retained in the ER does not trigger the transition of cells from the pro-B to the late pre-B stage (12, 60). Therefore, it is more likely that a pre-BCR acquires its signal competency after its transport to the cell surface, where it assembles immediately with BCR-specific signal molecules, such as tyrosine kinases and phosphatases, into a pre-BCR signalosome. Because the ratio of tyrosine kinases to phosphatases might differ between pre-BCR and BCR signalosomes (discussed in Ref. 6), a pre-BCR could, in contrast to its BCR counterpart, initiate constitutive signals without ligand binding. In this case, the only functions of the SL chain are 1) to initiate the transit of a pre-BCR from the ER to the cell surface by releasing a pre-BCR/SL chain complex (61), an ER-retained μH chain/Igαβ/Igαβ complex cannot be tyrosine phosphorylated in a pervandate model system (D. Mielenz and H.-M. Jäck, unpublished observations). In addition, a particular μ chain that is incapable to pair with A5 and is retained in the ER does not trigger the transition of cells from the pro-B to the late pre-B stage (12, 60). Therefore, it is more likely that a pre-BCR acquires its signal competency after its transport to the cell surface, where it assembles immediately with BCR-specific signal molecules, such as tyrosine kinases and phosphatases, into a pre-BCR signalosome. Because the ratio of tyrosine kinases to phosphatases might differ between pre-BCR and BCR signalosomes (discussed in Ref. 6), a pre-BCR could, in contrast to its BCR counterpart, initiate constitutive signals without ligand binding. In this case, the only functions of the SL chain are 1) to initiate the transit of a pre-BCR from the ER to the cell surface by releasing a pre-BCR/SL chain complex (61), an ER-retained μH chain/Igαβ/Igαβ complex cannot be tyrosine phosphorylated in a pervandate model system (D. Mielenz and H.-M. Jäck, unpublished observations).
prevents its efficient release from BiP. Consequently, those μH chains would be expressed on the cell surface at levels too low to initiating ligand-independent signal initiation. In this case, A5-mediated interaction of a pre-BCR with a stroma cell-associated HS chain could rescue signal initiation by concentrating the few pre-BCRs into signal-competent lipid rafts (45). Alternatively, a pre-BCR/HS interaction could recruit pre-B cells into a microenvironment with a high local concentration of HS-bound IL-7 (33). HS-bound IL-7 could then interact with its high affinity IL-7 receptor on the pre-B cell surface, which maintains, together with pre-BCR signals, the proliferation of pre-B cells. In support, pre-BCR positive pre-B cells require much less IL-7 for in vitro proliferation than pre-BCR negative pre-B cells (reviewed in Ref. 64).

In summary, we have shown that efficient interaction of a murine pre-BCR with ST2 stroma cells requires the unique non-Ig-like tail of A5 and a stroma cell-associated HS; or in other words, the A5 tail controls via its basic region the binding of a pre-BCR to an acidic HS chain on stroma cells. Whether the interaction between a pre-BCR and stroma cell-associated HS indeed induces signal transduction, and thus pre-B cell proliferation, remains to be further analyzed. However, with the findings reported in this study, we can now address this important question in appropriate experimental systems.

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References
13. ten Bosch, E., F. Melchers, and A. G. Rolink. 1998. Precursor B cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B cell receptor surface expression. Immunology 81:199.