Identification of Stromal Cell Products That Interact with Pre-B Cells

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Abstract. Our understanding of lympho-hematopoietic microenvironments is incomplete, and a new cloning strategy was developed to identify molecules that bind to B lineage lymphocyte precursors. A cell sorting procedure was used for initial enrichment of cDNAs from stromal cell mRNA that contained signal sequences and were therefore likely to encode transmembrane or secreted proteins. A second step involved expression of the library as soluble Ig fusion proteins. Finally, pools representing these proteins were screened for the ability to recognize pre-B cells. This approach resulted in the cloning of biglycan, syndecan 4, collagen type I, clusterin, matrix glycoprotein scl, osteonectin, and one unknown molecule (designated SIM). The full-length cDNA of SIM revealed that it is a type I transmembrane protein, and its intracellular domain has weak homology with myosin heavy chain and related proteins. Staining of established cell lines and freshly isolated hematopoietic cells with the Ig fusion proteins revealed distinct patterns of reactivity and differential dependence on divalent cations. Biglycan–, scl–, and SIM–Ig fusion proteins selectively increased interleukin 7–dependent proliferation of pre-B cells. Overexpression of the entire SIM protein affected the morphology of 293T cells, while expression of just the extracellular portion was without effect. Thus, a series of stromal cell surface molecules has been identified that interact with blood cell precursors. Three of them promoted the survival and/or proliferation of pre-B cells in culture, and all merit further study in relation to lympho-hematopoiesis.

Blood cell formation is highly dependent on complex interactions between stromal and hematopoietic cells (23). Multiple cell types transiently interact within a confined space in bone marrow to deliver and to receive critical signals for proliferation and differentiation. In addition, the movement of cells within, and egress from, this microenvironment is carefully linked to their maturation (1). It is important to identify cell surface molecules that mediate these interactions. Information already available suggests that several major categories of proteins are involved.

The extracellular matrix in bone marrow is not merely an inert framework; it also mediates specialized functions (33, 62). For example, it can bind many growth factors and cell surface glycoproteins. Characterization of substances made by stromal cells revealed that the matrix includes collagens, laminin, fibronectin, and a variety of proteoglycan species. Laminin is a major protein in basal lamina that can bind heparan sulfate, type IV collagen, and also cell surface receptor proteins. Fibronectin promotes migration and adhesion of many cells. Proteoglycans can attach to cell surfaces where they facilitate cell–matrix interactions. Hyaluronan forms viscous and hydrated gels, and in several systems, facilitates cell–cell adhesion and cell migration.

The large integrin superfamily of adhesion molecules includes many potential ligands for matrix molecules (15). Particular integrins on lympho-hematopoietic progenitors and stromal cells play an important role in hematopoiesis (23). Pre-B cells could be displaced from the adherent layer of long-term bone marrow cultures by addition of antibodies to α4 or (vascular cell adhesion molecule 1 (VCAM-1), and these reagents completely blocked lymphopoiesis in long-term bone marrow cultures (43, 44). While ligation of integrins can deliver signals for survival, growth, and differentiation, interactions between very late antigen (VLA)[1] 5 and fibronectin cause apoptosis in certain hematopoietic cell lines (58).

Multiple isoforms of CD44 constitute another family of cell adhesion molecules, and one of their ligands is hyaluronan (32). Antibodies to CD44 completely blocked the production of lymphoid and myeloid cells in long-term bone marrow cultures (42). Although most blood cells express CD44, only subpopulations actually use it to recog-

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1. *Abbreviations used in this paper*: IL, interleukin; PE, phycoerythrin; RT, reverse transcriptase; VLA, very late antigen.

nize hyaluronan, and this function is controlled in part by glycosylation of CD44 (18, 30). Interactions between CD44 and hyaluronan are important in extracellular matrix formation and for cell migration (26, 62).

Regulatory cytokines in the bone marrow microenvironment represent a final category and are essential for replication and differentiation of blood cell precursors (22, 33, 62). These factors are typically made in extremely small quantities, and some of them, such as interleukin (IL) 7 (IL7), IL3, and granulocyte-macrophage colony stimulating factor (G/M-CSF), are capable of attachment to the extracellular matrix. Certain other cytokines, such as macrophage colony stimulating factor and stem cell factor, are synthesized as transmembrane, as well as soluble, forms.

A number of experimental approaches have been used to implicate matrix molecules, cell adhesion molecules, and cytokines in blood cell formation, and there are advantages and limitations to each (23). While mAbs have been extensively and effectively exploited, highly conserved molecules may not be immunogenic. Furthermore, it can be difficult to prepare antibodies that block, and thus define, important functions. An alternative approach is suggested by the fact that a signal sequence is present during biosynthesis of most extracellular proteins, including type I transmembrane proteins and cytokines. Signal sequence trap methods exploit this fact to clone genes for such proteins (55, 59).

We established a new cloning strategy for identifying either cell surface or soluble molecules with the ability to recognize hematopoietic cells. A previously described signal sequence trap method (59) was modified and used for initial enrichment of cDNAs encoding transmembrane and secreted proteins. Pools from this enriched library were then expressed as soluble Ig fusion proteins, which were subsequently screened for binding to pre-B cells. This method allowed us to identify six extracellular matrix components and one previously unknown type I cell surface molecule. Furthermore, initial experiments suggest that some of these proteins might function to regulate blood cell formation.

Materials and Methods

Cells

BCB8, BCB9, and BCB10 pre-B cell clones were established from nonadherent cells of Whitlock-Witte–type long-term cultures that had been prepared from BALB/c bone marrow cells. These lymphocytes were maintained in McCoy's 5A medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FCS (GIBCO BRL) and 5×10^{-5} M 2-mercaptoethanol in the presence of 1 ng/ml IL7 (R&D Systems, Minneapolis, MN) and 10 ng/ml stem cell factor (Amgen, Thousand Oaks, CA). Cloned OP42 stromal cells were established using adherent spleen cells from osteopetrotic OP/OP B6C3fe mice (56). They lack the capacity to synthesize macrophage colony stimulating factor (M-CSF) and efficiently support the growth of B lymphocyte precursors when exogenous IL7 is added. These cells were maintained in DME supplemented with 10% FCS and 5×10^{-5} M 2-mercaptoethanol. Lymphoma cell lines (7OZ/3, WEHI231, AKR1, EL4), a B cell hybridoma (BM2), an erythroleukemia cell line (GM86), myelo-macrophage cell lines (WEHI3, P388D1), stromal cell lines (BMS2, ST2), fibroblast cell lines (NIH3T3, L), a melanoma cell line (B16F10), and a neuroblastoma cell line (N2A) were maintained as previously described (43). COS, an SV-40–transformed African green monkey kidney cell line, and 293T, a human renal carcinoma cell line transfected with large T antigen, were maintained in DME supplemented with 10% FCS.

Plasmids

To make NotI and XhoI sites, two oligonucleotides, 5′-CTCTAGATCTGCGGCCGCTGACTAACTGACCTCGAGG and 5′-GATCCCTCGAGGTCAGTTAGTCAGCGGCCGCAGATCTAGAGGTAG, were annealed and ligated into KpnI and BamHI sites of pBluescript KS to yield pBSKNotI/XhoI. To make the HPC4 epitope tag sequence, two oligonucleotides, 5′-TCGAGAGAAGATCAGGTAGATCCGCGGTTAATCGATGGTAAGATTGAAGGAAGGG and 5′-AATTCCCTTCCTTCAATCTTACCATCGATTAACCGCGGATCTACCTGATCTTCTC, were annealed and ligated into XhoI and EcoRI sites of pBluescript KS (HPC4/pBSK) (50). A plasmid containing the transmembrane domain of tissue factor (pJH93; donated by Dr. J.H. Morrissey, Oklahoma Medical Research Foundation, Oklahoma City, OK) was digested with EcoRI and BamHI before ligation into HPC4/pBSK (HPC4-TF/pBSK). HPC4-TF/pBSK was used as a template for PCR by two primers, 5′- CGCGGATCCGGAAGATCAGGTAGATCCGCGG and 5′-CGCAGATCTAAGCTTACTTGTGTAGAGA. This PCR product was digested with BamHI and BglII and ligated into the BamHI site of pBSKNotI/XhoI (HPC4-TF/pBSKNotI/XhoI). The pEFBOS vector (45) (a kind gift from Dr. S. Nagata, Osaka Bioscience Institute, Osaka, Japan) was modified by site-directed mutagenesis to remove the XhoI site at 3524, yielding pEFBOSX. This plasmid was then used as a recipient for the insert sequence of HPC4-TF/pBSKNotI/XhoI by using an XbaI site (HPC4-TF/pEFBOSX). A cDNA of CH2 and CH3 domains of human IgG_1 was digested at BamHI and BglII sites of the CD8-IgG1 vector (2) (generously provided by Dr. A. Aruffo, Bristol-Meyers Squibb) and ligated into a BamHI site of pBSKNotI/XhoI (Ig/pBSKNotI/XhoI). The insert sequence of Ig/pBSKNotI/XhoI was cut and ligated into the pEFBOSX vector by using an XbaI site (Ig/pEFBOSX). All constructs were confirmed by sequencing. The HPC4-TF/pEFBOSX vector was used for the signal sequence trap method. The Ig/pEFBOSX vector was used for producing Ig fusion proteins.

Stromal Cell cDNA Library

A cDNA library was made by converting poly $(A)^+$ RNA (5 μg) of OP42 stromal cells to cDNA with the use of random primers and SuperScript® reverse transcriptase (GIBCO BRL). A dC tail was then added to the cDNAs at the 3′ end with terminal deoxynucleotidyl transferase (GIBCO BRL). The second strands were synthesized by priming with 5′ GGTACCGCGGCCGCTGACTAACTGAC-$(dG)_{17}$, which contained a NotI linker and oligo dG. After sonication, DNA fragments >250 bp were isolated by electrophoresis in a 1% agarose gel. After blunting, the XhoI adaptors 5′-CCGCCTCGAGGATATCAAGCTTGTAC and 3′-GGCGGAGCTCCTATAGTTCGAACATGGAG were ligated. The fragments were then amplified by PCR with two primers, 5′-GGTACCGCGGCCGCTGACTAACTGACG and 5′-GAGGTACAAGCTTGATATCCTCGAGGCGG. 15 cycles of PCR reaction (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) were done with a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). Amplified DNA fragments were digested with NotI and XhoI and ligated into NotI and XhoI sites of the HPC4-TF/pEFBOSX vector.

Enrichment of cDNAs Carrying Signal Sequences

A positive control CD44 (5′) fragment including a signal sequence was obtained by NotI and XhoI digestion of PCR samples of the pMCD44/TCFMo plasmid (31) template with two primers, 5′-GGGGCGGCCGCACGCCATGGACAAGTTT and 5′-CGCCTCGAGTCTGGAATCTGAGGTCTC. A negative control BCL2 (5′) fragment that does not have a signal sequence was obtained by NotI and XhoI digestion of PCR samples of the pKSBcl2 plasmid template with two primers, 5′-GGGGCGGCCGCACTCGGGACTTGAAGTGCCATTGG and 5′-GGGCTCGAGGGAGACTGCCTGGCAGCCATCTCC. Both fragments were ligated into NotI and XhoI sites of the HPC4-TF/pEFBOSX vector.

The cDNA library contained in the HPC4-TF/pEFBOSX vector was transfected to *Escherichia coli*, and plasmid DNAs from all ($\sim 3 \times 10^5$) colonies were prepared. Pooled plasmid DNAs (20 μg) were then transfected into 5×10^6 COS cells by electroporation (250 V, 500 μF) using a gene pulser (Bio Rad Laboratories, Hercules, CA). The COS cells were stained with the HPC4 antibody and fluorescein-conjugated goat anti–mouse Ig 2 d after transfection, and cells that expressed the HPC4 epitope tag on their surface were collected by cell sorting. The plasmid DNAs were recovered from sorted cells by Hirt's method (14). For enrichment of cDNAs carrying signal sequences, this process was repeated three times.

Cloning Based on Binding of Expressed Proteins to Hematopoietic Cells

Plasmids from the signal sequence–enriched library were digested with NotI and XhoI, and then DNA fragments were isolated by gel electrophoresis and ligated into NotI and XhoI sites of Ig/pEFBOSX vector. After transformation of *E. coli*, 900 individual colonies were picked, and pools containing 10 colonies each were prepared. Plasmid DNAs from each pool were transfected into 293T cells with a calcium phosphate method. 4 d after the transfection, supernatants were harvested from each sample. BCB10 cells were then stained with these supernatants and fluorescein-conjugated goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL). After identification of a positive pool, each clone of that pool was rescreened. The nucleotide sequence of each cloned insert was determined by using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH). Nucleotide database searching was then performed with FASTA and BLAST from the GCG computer program (Genetics Computer Group, Madison, WI).

Cloning of Full-Length 4-9 cDNA

To perform PCR, sense and antisense primers, 5′-GGTGTCTACAGCAGGCGCATTGTTG and 5′-GGTCTTCCCTTAGGAACTCATCAC, were designed from the 5′ portion cDNA insert sequence of 4-9. As BMS2 stromal cells also express this gene, a cDNA library was made in the pEFBOS vector from mRNA of BMS2 cells using oligo dT and a TimeSaver cDNA synthesis kit (Pharmacia, Uppsala, Sweden). Suspensions of pools containing several thousand colonies were subjected to PCR reactions with the above two primers. After 35 cycles of PCR reaction (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min), PCR samples were separated in 1.5% agarose gels and screened with a specific amplified band (327 bp). A positive pool was divided into progressively smaller pools and rescreened until a single clone was isolated. The insert of a single clone was subcloned into pBluescript, and the nucleotide sequence was determined using an automated DNA sequencer (Applied Biosystems, Foster City, CA).

Flow Cytometry Analysis

Antibody incubations and washing steps were perfomed at room temperature in HBSS (GIBCO BRL) containing 1% BSA (Sigma Chemical Co., St. Louis, MO) and 0.1% sodium azide, or in 10 mM Tris-HCl saline containing 1% bovine albumin, 0.1% sodium azide, and 5 mM $MnCl_2$. Cells were analyzed with a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA). Potential binding to FcγII and FcγIII receptors was blocked in some experiments by addition of the 2.4G2 mAb (61). As another means of minimizing possible recognition of Fc receptors, a mutated CH2 + CH3 cassette of human IgG_1 (37), obtained from Dr. Peter Linsley (Bristol-Myers Squibb), was used for expression of soluble Ig fusion proteins in some experiments. Supernatants of cultures of 293T cells transfected with the Ig/pEFBOSX vector, as well as soluble CD44–Ig, CD73–Ig, and CD7–Ig fusion proteins, were used as negative controls. Antibodies used in this study were as follows: phycoerythrin (PE)–conjugated anti–mouse CD45RA from Pharmingen (San Diego, CA); FITC-conjugated anti–mouse Mac1 from Boehringer Mannheim Biochemicals (Indianapolis, IN); PE-conjugated anti–mouse IgM and FITC- or PE-conjugated goat $F(ab')_2$ anti–human IgG (mouse adsorbed); and FITC-conjugated goat anti–human IgG from Southern Biotechnology Associates.

Reverse Transcriptase (RT)–PCR

Total RNAs were isolated by the method of Chomzynski and Sacchi (5). Total RNA (5 μg) was reverse transcribed to cDNA in a total reaction volume of 20 μl comprised of Moloney leukemia virus reverse transcriptase (Promega, Madison, WI), oligo dT (0.5 μg), 0.1 M DTT, 10 mM each dNTP, and 1× RT buffer. The RT mixes were incubated at 39°C for 2 h followed by 10 min at 90°C. To perform PCR reactions, 2 μl of the above RT mixtures were added to PCR buffer containing $MgCl_2$ (1.5 mM), Taq polymerase (1 U; Promega), dNTP (2 mM each), and relevant sense and antisense primers to a total volume of 100 μl. PCR reaction mixtures were amplified by using 30 cycles under the following conditions: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min in a thermal cycler. PCR samples (15 μl) were electrophoresed in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. Products were visualized under UV illumination. The oligonucleotide primers used for these reactions were as follows:

Biglycan	Sense:TACCTTCAGTGCCATGTGTCCT
	Antisense:CTGGTTCAAAGCTGTTCTCC
Syndecan 4	Sense:CCGCTGCTGCTGCTGCTCCTCGGA
	Antisense:GCATTCTCAGGGATCTGGTTATCC
Collagen I	Sense:GTCTTACTGGGAACTTTGCTGCTC
	Antisense:GCCAACGCTTCCATCACTGCCTCG
Clusterin	Sense:AGCTCCAAGAACTGTCCACTCAAG
	Antisense:GACATGAGGCTGCGGACCAAGCGG
Osteonectin	Sense:AGACTGAAGTTGCTGAGGAGATAG
	Antisense:AGAGGGAATTCGGTCAGCTCGGAA
scl	Sense:CTTCAAGCTACCAAGGCTCTGGAT
	Antisense:CCTGCTCTGTTGACTGTTCATGAG
4-9	Sense:GGTGTCTACAGCAGGCGCATTGTTG
	Antisense:GGTCTTCCCTTAGGAACTCATCAC
β-Actin	Sense:CCTAAGGCCAACCGTGAAAAG
	Antisense:TCTTCATGGTGCTAGGAGCCA

Northern Blot Analysis

A blot containing 2 μg of poly(A)$^+$ RNAs from adult mouse tissues was acquired from Clontech (Palo Alto, CA). The 4-9– and β-actin–containing cDNA fragments were labeled with [α-^{32}P]dCTP using a random primed DNA labeling kit (Boehringer Mannheim Biochemicals) and hybridized to the membrane overnight at 42°C. Blots were then washed and autoradiographed.

Colony-forming Cell Assay

For the enrichment of B lymphocyte lineage cells, bone marrow cells from 6–8-wk-old BALB/c mice were incubated in mAb 14.8 (CD45RA, a pan B lineage marker) coated petri dishes for 40 min at 4°C as in our previous studies (40). After nonbinding cells were washed off, bound cells were recovered by pipetting. Whole bone marrow cells or the lymphocyte-enriched cells were suspended in 1 ml of assay medium, and the semisolid agar colony assay for B lymphocyte precursors (CFU-IL7) was performed with 1 ng recombinant mouse IL7. The granulocyte-macrophage progenitor assay (CFU-c) was similarly performed with 25 μl of 10-fold concentrated WEHI3 conditioned medium. All colony assays were perfomed in 35-mm dishes and incubated at 37°C, 5% CO_2. Numbers of colonies were counted on day 6. The fusion proteins used in titration experiments (see Fig. 6) were purified on protein A–bearing affinity columns (Pierce Chemical Co., Rockford, IL).

Results

Successful Enrichment of cDNAs Containing Signal Sequences

Signal sequence trap procedures previously have been used to identify cDNAs for extracellular or transmembrane proteins (59), and we now demonstrate that a cDNA library can be enriched on this basis. The HPC4-TF/pEFBOSX vector was constructed with NotI and XhoI cloning sites positioned between a human EF-1α promoter and coding sequences for the HPC4 epitope tag and the transmembrane domain of tissue factor (Fig. 1 *A*). When inserts with signal sequences were cloned in-frame with the correct orientation, fusion proteins were expressed on mammalian cells and detected by an antibody to the HPC4 epitope tag. In contrast, when inserts lacked signal sequences or were cloned out-of-frame, the fusion proteins remained intracellular. The effectiveness of this system was evaluated with a 5′ cDNA fragment of CD44 containing a signal sequence as a positive control. The HPC4 epitope tag appeared on the surface of 293T cells transfected with CD44-HPC4-TF/pEFBOSX (Fig. 1 *B*). A 5′ cDNA fragment of BCL2 that lacked a signal sequence was used as a negative control, and transfection with BCL2-HPC4-TF/pEFBOSX did not direct an epitope-tagged protein to the cell surface.

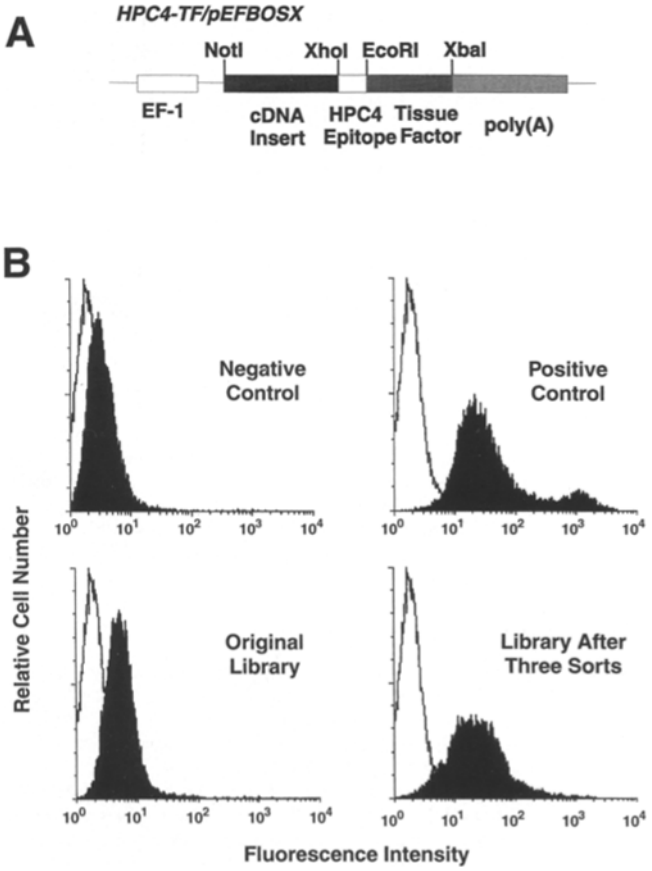

Figure 1. Enrichment of cDNAs containing signal sequences by repeated cycles of transfection and cell sorting. (*A*) The HPC4-TF/pEFBOSX vector was used for enrichment of cDNAs carrying signal sequences. (*B*) Negative control (BCL2-HPC4-TF/pEFBOSX), positive control (CD44-HPC4-TF/pEFBOSX), and cDNA-containing plasmids from different stages of the enrichment (library before and after sorting three times) were transfected into 293T cells using a Ca^{++} phosphate method. The transfected cells were then stained with an antibody to the HPC4 epitope tag. Shaded histograms depict staining obtained with the HPC4 mAb, and open histograms represent results obtained with the second antibody alone.

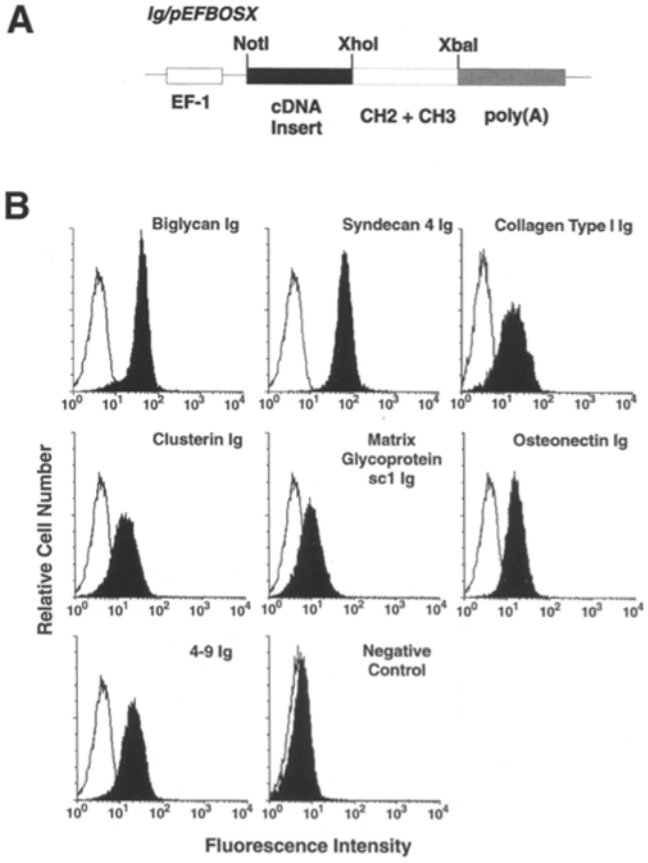

Figure 2. Recognition of a pre-B cell line, BCB10, by Ig fusion proteins. (*A*) The Ig/pEFBOSX vector was used to produce Ig fusion proteins. Each cloned plasmid and a control plasmid (CD44-Ig/pEFBOSX) were transfected into 293T cells. (*B*) BCB10 cells were then stained with supernatants from each transfected sample, followed by FITC-goat anti–human IgG (*shaded histograms*). HBSS containing 1% BSA and 0.1% azide was used as washing buffer. Control staining obtained with the second antibody alone is also shown (*open histograms*).

This vector was used to prepare a cDNA library from the OP42 stromal cell clone, and the average size of the inserts was 400 bp (range 250–600 bp). We transfected the library into COS cells and recovered plasmids from HPC4-positive cells that had been isolated by cell sorting. This procedure was repeated three times. The effectiveness of enrichment was monitored by flow cytometry and is illustrated in Fig. 1 *B*. Transfectants prepared from the repeatedly sorted library showed much higher expression of the HPC4 epitope tag than transfectants made with the original stromal cell cDNA library. Therefore, we successfully designed a system for enrichment of cDNA fragments that include sequences for signal peptides.

Isolation of Clones Whose Ig Fusion Proteins Recognize a Pre-B Cell Line

cDNAs that have been enriched for signal sequences would correspond to a host of proteins, and a second step was used to identify ones that may interact with pre-B cells. We designed the Ig/pEFBOSX vector, which directs production of soluble Ig fusion proteins. This vector had NotI and XhoI cloning sites, between the EF-1α promoter and coding sequences of the CH2 and CH3 domains of human IgG1 (Fig. 2 *A*). Thus, cDNAs with the correct reading frame in the signal sequence–enriched library (Fig. 1 *A*) would also be able to express an Ig fusion protein when subcloned into the Ig/pEFBOSX vector (Fig. 2 *A*). As a positive control, a construct was prepared with a cDNA containing the extracellular domain and signal sequence of CD44 in Ig/pEFBOSX, and 2 μg of this plasmid was transfected to 293T cells in 24-well plates. Supernatants from the transfectants were harvested on day 5 and found to contain ~10 μg/ml of CD44–Ig fusion protein (data not shown). The sensitivity of detection of particular clones was then tested by serial dilution experiments in which mixtures were made of the CD44-Ig/pEFBOSX and Ig/pEFBOSX plasmids. Supernatants containing CD44–Ig were recognized by the ability to bind to the hyaluronan-bearing BMS2 stromal cell clone. This revealed that one

Table I. A Summary of cDNA Clones Isolated with this Strategy

Molecule	Number of clones	Signal sequence	Extracellular	Insert cDNA
				bp
Biglycan	5	+	Yes	432
Syndecan 4	2	+	Yes	306
Collagen type I	2	+	Yes	384
Clusterin	1	+	Yes	264
Osteonectin	3	+	Yes	372
Matrix glycoprotein sc1	3	+	Yes	387
4-9 (unknown)	1	+	Yes	347
Saposin	1	+	No	ND

positive clone could be identified when it was diluted in a pool with 10 negative clones.

We picked 900 individual colonies from the signal sequence–enriched library in the Ig/pEFBOSX vector and made pools that each represented 10 colonies. Flow cytometry was then used to identify pools that contained plasmids capable of expressing fusion proteins that recognized BCB10 cells (Fig. 2 *B*). This pre-B cell line was used because it can be grown in the presence of IL7 plus stem cell factor, and also because it has a very low level of Fc receptor expression. A total of 18 individual clones whose Ig fusion proteins had this property were isolated and sequenced. All inserts contained 5′ terminal cDNA sequences, and their lengths varied from 264 to 432 bp (Table I). One encoded a known intracellular protein, saposin, and was not considered further for that reason (60). However, this protein is heavily glycosylated and also has a hydrophobic domain at the amino terminus, which presumably accounted for its selection with our method. One, 4-9, represented a previously unknown molecule. The others corresponded to previously described components of the extracellular matrix (biglycan, syndecan 4, collagen type I, clusterin, osteonectin, and matrix glycoprotein sc1) (9, 38, 47, 49). Each of these are known to have signal sequences and to be either secreted or expressed on the surface of cells. A murine homologue of matrix glycoprotein sc1 had not been previously cloned, and the sequence identified here had 80.6% identity to that described for the rat (16). Similarly, this represents the first isolation of murine syndecan 4, which has 91.5% identity to the rat homologue (19). Therefore, this cloning strategy did indeed select for cDNAs with signal sequences, and stromal cell molecules that are potentially relevant for interactions with pre-B cells were identified.

Isolation of the 4-9 cDNA Coding Region and Primary Structure of the Predicted Protein

The entire coding region of the 4-9 cDNA was isolated to permit more thorough homology searches and structural predictions. This was done with sense and antisense primers designed from the original 5′ cDNA sequence of 4-9, which were used to screen a stromal cell library by PCR (Materials and Methods). The originally isolated fragment of 4-9 was completely identical to the sequence between 133 and 479 of the full-length cDNA (Fig. 3; EMBL/GenBank/DDBJ accession number U47323). The latter 3,192-bp cDNA contains an open reading frame of 2,055 bp, which is flanked by 5′- and 3′-untranslated regions of 193

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  1 MDVCARLALW LLWGLLLHQG QSLSHSHSEK
 31 NTGASSGATS EESTEAEFCR TDKPLCHSED
 61 EKLSFEAVRN IHKLMDDDAN GDVDVEESDE
 91 FLREDLNYHD PTVKHSTFHG EDKLISVEDL
121 WKAWKSSEVY NWTVDEVIQW LITYVELPQY
151 EETFRKLQLT GHAMPRLAVT NTTMTGTVLK
181 MTDRSHRQKL QLKALDTVLF GPPLLTR**HNH
211 LKDFMLVVSI VIGVGGCWFA YIQNRYSKEH
241 MKKMMKDLEG LHRAEQSLHD LQERLHKAQE
271 EHRTVEVEKV HLEKKLRDEI NLAKQEAQRL
301 KELREGTENE RSRQKYAEEE LEQVREALRK
331 SEKELESHSS WYAPEALQKW LQLTHEVEVQ
361 YYNIKKQNAE RQLLVAKEGA EKIKKKRNTL
391 FGTFHVAHSS SLDDVDHKIL TAKQALSEVT
421 AALRERLHRW QQIEILCGFQ IVNNPGIHSL
451 VAALNIDPSW MGSTRPNPAH FIMTDDVDDM
481 DEEIVSPLSM QSPSLQSSVR QRLTEPQLGL
511 GSQRDLTHSD SESSLHMSDR QRVAPKPPQM
541 GRAADEALNA MPSNGSHRLI EGVHPGSLVE
571 KLPDSPALAK KTFMALNHGL DKAHSLMELN
601 PSVPPGGSPL LDSSHSLSPS SPDPDTPSPV
631 GDNRALQGSR NTRIPHLAGK KAMAEEDNGS
661 IGEETDSSPG RKKFPLKIFK KPLKK
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Figure 3. Amino acid sequence of full-length 4-9. A potential signal peptide is marked (*box*), but the actual cleavage site is unknown. The protein encoded by the original cDNA (*underlined with dashes*) and a possible transmembrane domain (*underlined.*) Potential N-glycosylation sites (*circles*) and a possible enzymatic cleavage site (*asterisks*). This sequence data is available from EMBL/GenBank/DDBJ under accession number U47323.

and 944 bp. The hydrophobicity profile of the deduced 685–amino acid protein predicts a possible NH_2-terminal signal peptide. This hydrophobic region precedes a possible signal cleavage site between Ser-22 and Leu-23, as predicted by the method of Heijne (12). A second hydrophobic stretch of 22 amino acids is located between positions 213 and 234, and could correspond to a transmembrane domain (8). The protein possesses a dibasic sequence before this segment, which could serve as a proteolytic cleavage site for release from the cell surface. There is also a single cysteine residue in the putative transmembrane domain that may allow interaction with other membrane proteins, and there are two potential sites for N-glycosylation at positions 131 and 171.

A computer search revealed that segments of the 4-9 sequence had been cloned before as part of genome projects, but this was not instructive as to its function. One previous isolation was from a human brain cDNA library (WashU-Merck EST Project, unpublished data; computation performed using the BLAST network service). Another represented only the 3′-untranslated region of 4-9 and was isolated from a murine teratocarcinoma cell cDNA library (46). The predicted extracellular portion of this protein had no significant homology to any other proteins. However, there was weak similarity between the intracellular portion of 4-9 and human myosin heavy chain (18.7% identity in a 198–amino acid overlap), human restin (15.2% identity in a 329–amino acid overlap), *Drosophila melanogaster* paramyosin (13.8% identity in a 441–amino acid overlap), and human centromeric protein E (14.4% identity in a 416–amino acid overlap). A predicted coiled-coil structure is a common feature of all of these proteins.

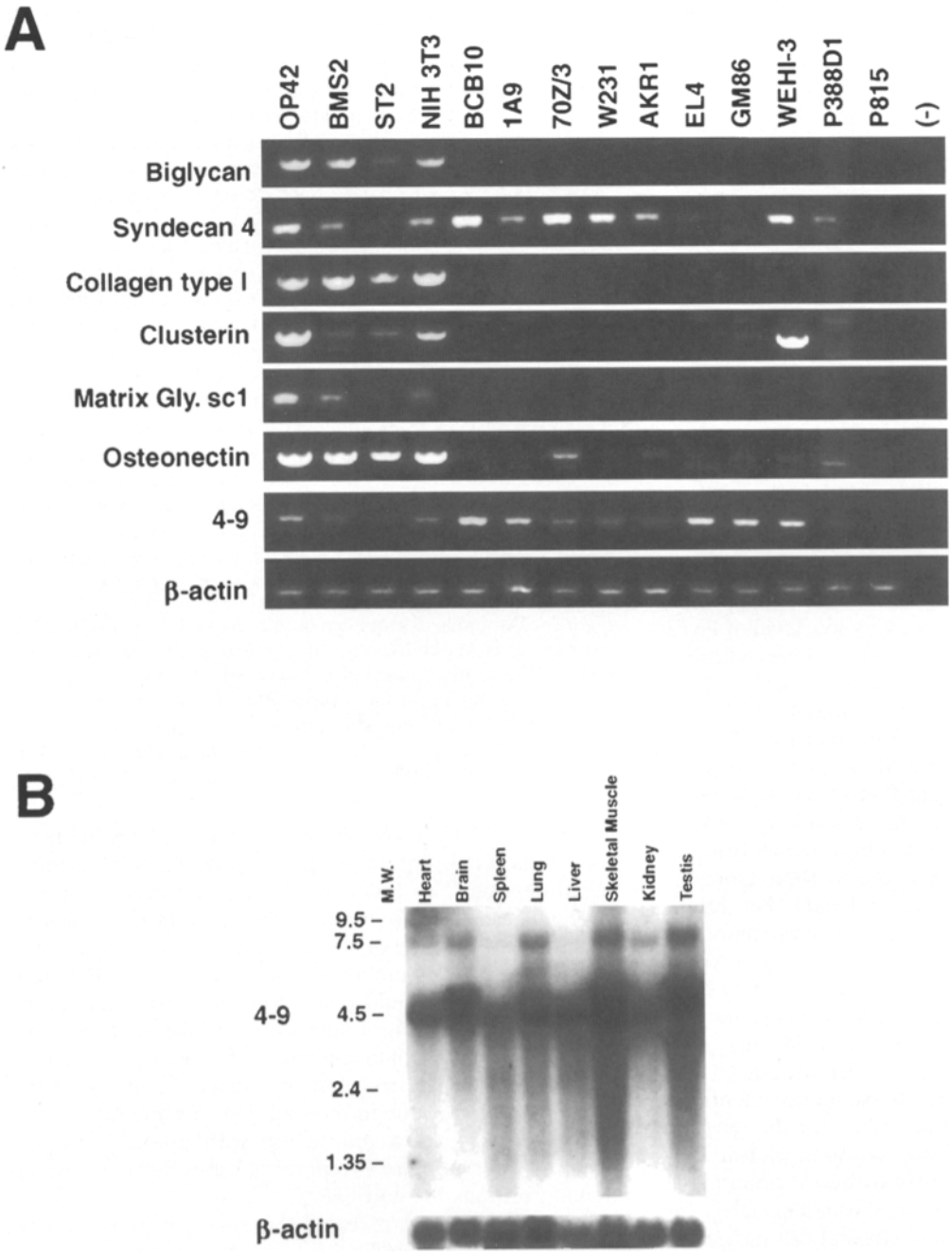

Figure 4. Expression of stromal cell products in various cells and tissues. (*A*) Total RNAs were isolated from the indicated cell lines and subjected to RT-PCR. The amplified products were electrophoresed through a 1% agarose gel containing ethidium bromide. Sequences for the oligonucleotide primers used for these reactions are given in Materials and Methods. (*B*) Poly(A)$^+$ RNAs (2 μg per lane) were from the indicated tissues, and the resulting blot was hybridized overnight to a probe containing the entire 4-9 cDNA sequence. (*Lower panel*) Control for equal loading where the same blot was probed with β-actin.

Expression of Stromal Cell Products in Various Tissues

Our cloning strategy should not necessarily select for genes that are expressed in a stromal cell–restricted fashion, and, indeed, this was not the case. Reverse transcriptase–PCR was used to survey expression by a panel of cell lines (Fig. 4 *A*), and the specificity of these reactions was confirmed by sequencing of the PCR products (data not shown). This analysis revealed that two of the genes we cloned (syndecan 4, 4-9) were expressed by many cell types, which included lymphomas and myeloid tumors, but not a mastocytoma (P815). Transcripts for the 4-9, but not syndecan 4 gene, were also detectable in an erythroleukemia cell line (GM86). Clusterin and osteonectin were each expressed by stromal cells and some lympho-hematopoietic cells. A more restricted pattern was observed with biglycan, collagen type I, and matrix sc1, as transcripts were only detectable in stromal cells and fibroblasts. Northern blot analysis confirmed that the previously unknown 4-9 gene is expressed in many tissues, but that there was some heterogeneity in transcript size (Fig. 4 *B*). The most prominent band was ~4.5 kb, but shorter and longer exposures consistently revealed minor species that were larger (9.5 and 5 kb) and smaller (3.7 kb).

Distribution of Ligands for Soluble Stromal Cell–derived Fusion Proteins

Experiments were then performed to evaluate interactions of stromal cell–derived fusion proteins with various types

Table II. Recognition Specificity of Expressed Ig Fusion Proteins as Evaluated by Staining of Established Cell Lines

Cell type	Cell line	Ig fusion proteins						
		Biglycan	4-9	Syndecan 4	Coll. 1	Clusterin	Scl	Osteonectin
Pre B	BCB10	++	+	++	++	+	±	+
Pre B	BCB8	+	±	+	++	±	±	±
Pre B	BCB9	++	+	++	++	+	±	+
B Lymphoma	WEHI231	++	−	++	−	−	−	−
B Hybridoma	BM2	±	−	±	−	−	−	−
T Lymphoma	EL4	−	−	−	−	−	−	−
T Lymphoma	AKR1	−	−	−	−	−	−	−
Myeloid	WEHI3	±	±	±	+	+	±	±
Erythroleukemia	GM86	+	+	++	−	−	−	+
Stromal	OP42	+	−	+	−	−	−	−
Stromal	BMS2	+	−	±	−	−	−	−
Fibroblast	L Cells	+	−	±	−	−	−	−
Neuroblastoma	N2A	+	−	+	−	−	−	−
Melanoma	B16F10	+	−	+	−	−	−	−

++, >90%; +, >50%; ±, >10%; and −, <10% of cells staining.

of lympho-hematopoietic cells. Two steps were taken to minimize the possibility of recognition via Fc receptors. All of the soluble proteins were made with a human IgG_1 cassette that had been mutated to diminish Fc-mediated binding (37) (see Materials and Methods), and in some experiments, cells were pretreated with an mAb (2.4G2) to Fc receptors. The latter treatment had no influence on fusion protein staining of BCB10 cells, but did slightly reduce the background recognition of $CD11b^+$ myeloid cells (data not shown). An example of recognition of the BCB10 pre-B cell clone is illustrated in Fig. 2 *B*, where a CD44–Ig fusion protein is included as a negative control. Similar levels of background staining were observed when human CD73–Ig or CD7–Ig were used as control proteins (data not shown). The intensity of staining was consistently strongest with biglycan and syndecan 4 fusion proteins. Recognition of two other B lineage lymphocyte precursor clones (BCB8 and BCB9) was similar to that observed with BCB10, and two of the fusion proteins gave low, but significant, staining when tested on an immature B lymphoma (WEHI231; Table II). In contrast, none of the fusion proteins recognized either of two T lymphomas (AKR1 and EL4). Biglycan and syndecan 4 fusion proteins weakly stained a number of cell types, but recognition was very limited with the other fusion proteins.

Conditions for Recognition of Normal Lympho-hematopoietic Cells

Divalent cations facilitated recognition of BCB10 cells by fusion proteins prepared with 4-9, biglycan, collagen type I, clusterin, matrix glycoprotein scl, and osteonectin, as staining was completely blocked by addition of EDTA (Fig. 5 *A*; data not shown). This treatment also slightly diminished staining with the syndecan 4–Ig fusion protein (data not shown). It has been shown that Mn^{++} substantially increases the ability of integrins to recognize ligands (39). We found that recognition of pre-B cells with 4-9 and osteonectin also increased with this treatment (Fig. 5 *A*; data not shown). Antibodies to α4 (PS/2), α5 (MFR5), α6 (GoH3), α_L (FD441.8 and M17/4.2), β1(KMI6 and 9EG7), or β2 (M18/2) did not block the binding of 4-9–Ig to BCB10 cells (data not shown). Moreover, 4-9–Ig did not recognize BAF3 cells even in the presence of Mn^{++}, although this line expresses VLA4, VLA5, VLA6, and CD11a (data not shown). Thus, the counter-receptor/ligand for 4-9 on BCB10 cells was not readily attributed to any of these integrins. Addition of heparin significantly blocked staining by the 4-9 fusion protein, a finding that could also be instructive about its counter-receptor.

All of the above studies used lympho-hematopoietic tumors and cultured cell lines. Therefore, we used two-color flow cytometry to evaluate the ability of the 4-9–Ig fusion protein to interact with freshly isolated bone marrow cells (Fig. 5 *B*). This protein bound to 51% of marrow cells, and 68% of the positive cells also expressed the B lineage marker CD45R. Only weak staining was observed with ~8% of bone marrow–nucleated cells using the control CD44–Ig fusion protein, and these did not represent B lineage cells. The ability of lymphocyte precursors to interact with 4-9 appeared to increase with differentiation, and 78% of cells that had acquired sIgM (B cells) were stained by this fusion protein. Similarly, 88% of mature B cells in the spleen were recognized (data not shown). While the 4-9 fusion protein recognized lymphocytes and their precursors in marrow, it was not lineage specific, as 9–36% of myeloid cells (assessed with Ly-6C/GR1 or CD11b/Mac-1, respectively) and 55% of erythroid cells ($TER119^+$) were stained. In addition, 20% of splenic T cells were recognized (data not shown). These analyses were performed in the presence of Mn^{++}, and, as with cell lines, failure to add this divalent cation resulted in much weaker staining. These findings suggest that a counter-receptor/ligand for the 4-9 stromal cell product is present on normal hematopoietic cells and may increase in density with B lymphocyte differentiation.

Influence of Stromal Cell Products on B Cell Precursors

Production of B lineage lymphocytes is dependent on IL7, and numbers of IL7-responsive precursors can be enumerated with a semisolid agar cloning assay (29). This was used in a preliminary evaluation of possible functions for

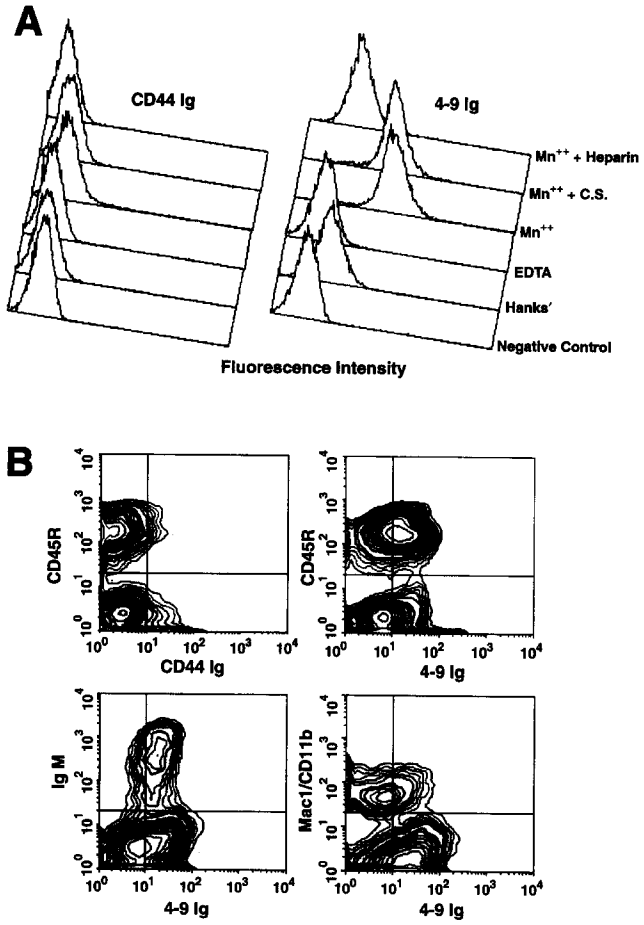

Figure 5. The 4-9–Ig fusion protein recognizes normal cells, and the binding requires divalent cations. (*A*) BCB10 cells were stained with the 4-9–Ig fusion protein (*right*) in the presence of Hank's solution (1.3 mM Ca^{++}, 0.9 mM Mg^{++}), Hanks with EDTA (5 mM), Tris buffer with Mn^{++} (5 mM), Tris buffer with both Mn^{++} and chondroitin sulfate (*C.S.*, 5 μg/ml), or Tris buffer with both Mn^{++} and heparin (5 μg/ml). Background staining with the second antibody alone is also shown (*right*; *Negative Control*), along with staining obtained with a control CD44–Ig fusion protein (*left*). (*B*) Two-color analysis of bone marrow included markers of B lineage lymphoid cells (*CD45R* and surface *IgM*) and myeloid cells (*Mac1/CD11b*). Quadrants are indicated to show levels of background staining (obtained with irrelevant isotype-matched antibody or second antibody alone controls). Staining and washing were done in the presence of 5 mM $MnCl_2$.

the molecules we identified. Soluble Ig fusion proteins representing CD44, syndecan 4, osteonectin, or collagen type I had no significant effect on lymphocyte proliferation when added to these cultures (Fig. 6; data not shown). However, the cloning efficiency was increased upon addition of biglycan-, scl-, or 4-9–containing fusion proteins. This ranged from 23–76% elevation in six individual experiments, and was similar for the original version of 4-9 and a fusion protein that contained the entire extracellular sequence. None of the proteins elicited colony formation when added to semisolid agar cultures in the absence of IL7 (data not shown). As little as 2 μg of the 4-9–Ig fusion protein influenced clonal proliferation of B cell precursors ($P = 0.0015$; Fig. 6). Similar dose responsiveness was demonstrated with biglycan and scl, but 10 times this amount

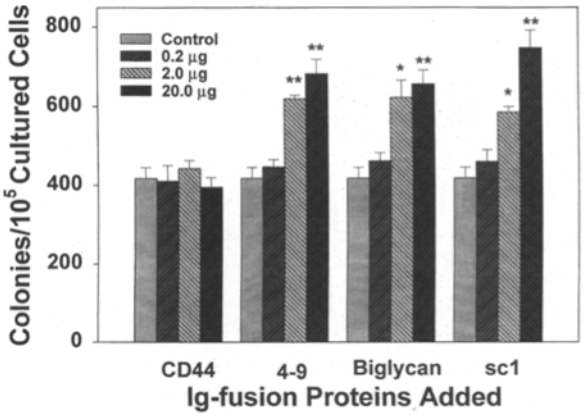

Figure 6. Dose-dependent augmentation of B cell precursor expansion by Ig fusion proteins. Biglycan–Ig, 4-9–Ig, scl–Ig, and CD44–Ig proteins were purified on protein A columns and added to CFU-IL7 colony assays at the indicated concentrations. Results represent mean numbers of colonies per 10^5 cultured cells ± SEM ($n = 3$). Statistically significant differences from control (CD44–Ig) values are indicated by one ($P \leq 0.05$) or two ($P \leq 0.01$) asterisks.

of the control CD44–Ig fusion protein had no significant effect. Myeloid progenitors (CFU-c) that responded to colony stimulating factor were not significantly influenced by any of the Ig fusion proteins (data not shown). Therefore, some of the stromal cell–derived products promote expansion of IL7-responsive lymphocyte precursors.

Morphological Changes Resulting from Overexpression of the 4-9 Protein

No remarkable changes were observed in the growth or appearance of 293T cells transfected with the originally isolated 4-9 cDNA (14% of the total sequence plus the epitope tag and transmembrane domain of tissue factor). This was also true when the entire extracellular portion was overexpressed with the tissue factor transmembrane domain (data not shown). However, cells expressing the full-length sequence assumed a rounded morphology and detached from the dish within 2 d of transfection (Fig. 7). Since the change occurred before confluence was reached, it did not result from overgrowth of contact-inhibited cells. While numbers of cells recovered 3–4 d after transfection with this plasmid were slightly reduced, there was no effect on cell viability. This result suggests that the native 4-9 protein may have roles in adhesion and/or cell shape that require the transmembrane and/or cytoplasmic domains.

Discussion

We have described a new cloning strategy and its use to identify a series of stromal cell genes whose products interact with pre-B cells. An enrichment procedure was devised for cDNAs that encode type I transmembrane or secreted proteins with signal peptides. A second step involved expression of the resulting library as soluble Ig fusion proteins and screening for ones that recognized lymphocytes. While there are advantages and limitations to any approach, this one was successfully used to identify

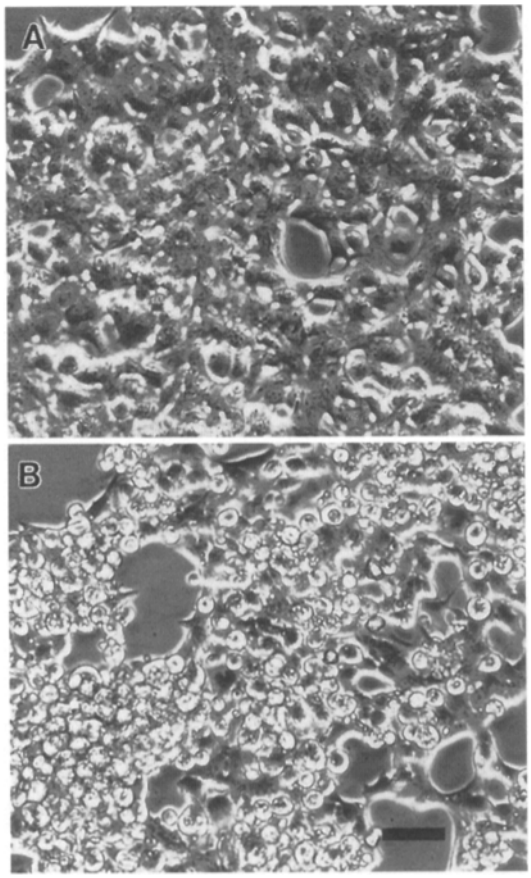

Figure 7. Rounding and detachment of 293T cells after overexpression of the full-length cDNA of 4-9. Phase-contrast micrographs are shown of 293T cells transfected with pEFBOS (control plasmid) (*A*) or the full-length native 4-9/pEFBOS (*B*). Bar, 50 μm.

several components of the bone marrow microenvironment that may prove to be important in regulation of blood cell formation.

Honjo and colleagues reported that it was possible to selectively clone cDNAs corresponding to secreted or transmembrane molecules (59). As in their method, we incorporated an epitope sequence in the expression vector. However, we then expressed the resulting library in COS cells and used an mAb to the epitope tag with cell sorting to enrich for cDNAs that included signal sequences. We used COS cells and electroporation at this stage with the aim of obtaining relatively low copy numbers in the transfected cells. There are two indications that this enrichment step was successful. Transfection of the same amount of plasmid DNA yielded progressively higher expression of the epitope tag after the three cycles of sorting (Fig. 1 *B*). Secondly, clones of interest were relatively abundant in the enriched library, as 1.8% of the clones yielded an Ig fusion protein that recognized pre-B cells. In preliminary experiments, we determined that a pool size of no more than 10 clones per pool was required to detect binding of a CD44–Ig control protein to a hyaluronan-bearing cell. Therefore, we doubt that it would have been feasible to search for relevant molecules expressed as Ig fusion proteins without prior enrichment of the library for cDNAs that encode signal sequences.

Our objective in screening Ig fusion proteins expressed by the stromal cell library was to identify important molecules that might have been overlooked by other experimental approaches. For example, highly conserved cell interaction molecules might not be immunogenic, making it difficult to produce mAbs against them. Also, screens based on cell adhesion could require relatively high avidity binding or cooperation between multiple molecules. Our procedure yields sequences for individual molecules with potentially interesting recognition properties, and the necessary threshold of binding affinity could be lower than with other methods. It produces soluble cell interaction molecules with defined recognition specificity that can be used in functional and other assays. The binding can also provide useful information about the nature of the counter-receptor (e.g., requirement for divalent cations, blocking by heparin) and its representation in various tissues. Furthermore, this method is readily adaptable to detect other kinds of cell interaction molecules. For example, we could screen the same enriched library for proteins that specifically recognize hematopoietic stem cells. In principal, any pair of interacting cells could be used to derive cDNA libraries and for screening in either direction. We selected stromal cells for library preparation rather than lymphocytes because staining of the latter was more efficiently evaluated by flow cytometry.

A relatively small insert size (~400 bp) was used to avoid the possibility that transmembrane domains would be cloned 5′ of the epitope tag. Therefore, we would not have identified proteins whose entire length is required for function, and it is notable that no cytokine genes were cloned. We subsequently obtained full-length (or entire extracellular portions) cDNA sequences for three of the clones (4-9, syndecan 4, and osteonectin). When expressed as Ig fusion proteins, they recognized pre-B cells as well as the original ones, which only contained partial sequences. Therefore, we may have preferentially identified proteins whose amino-terminal portions were sufficient for binding to pre-B cells. Similarly, this method would not isolate molecules that only function as part of complexes. Transfectants were prepared that initially contained plasmids representing ~10 clones, and this would produce a number of heterodimeric Ig fusion proteins. These might have lower affinity than homodimers, and detection of relatively abundant proteins could be favored. On the other hand, the enrichment achieved with the first step of our approach should at least partially diminish this as a problem. As another possible limitation, posttranslational modifications of molecules expressed in 293T cells might differ from those typical of stromal cells. We would not have identified proteins whose recognition specificity was influenced in this way.

All but one of the stromal cell products we identified have been previously studied, and there are indications that some of these molecules may participate in lymphohemopoiesis. For example, an inhibitor of collagen synthesis diminished production of hematopoietic cells in long-term bone marrow cultures (64). Multiple collagen types can be made by bone marrow stromal cells, and precursors of myeloid and erythroid cells have been shown to adhere to collagen type I (27, 62). There are four known types of syndecan, and they are known to be expressed in a tissue-

and differentiation-specific fashion (20). Syndecan 1 is present on pre-B cells, down-regulated as they mature, and reacquired at the plasma cell stage (53). This molecule has been shown to mediate adhesion of plasmacytoma cells to collagen type I (54), but we do not yet know if it is involved in pre-B cell recognition of stromal cell–derived collagen. Overexpresssion of syndecans 1 and 4 in B lymphoid cells can trigger aggregation, suggesting again that these molecules may be involved in cell recognition (57). Osteonectin has also been implicated in cell adhesion. While osteonectin can mediate the aggregation of platelets, it can be antiadhesive in other circumstances (28). Osteonectin is detectable in a granular pattern on adherent cells in long-term bone marrow cultures (34).

Biglycan was previously isolated from the thymus and shown to function as a macrophage colony–stimulating factor (17). Only 91 of the amino-terminal residues of biglycan (~25% of the molecule) were present in our Ig fusion protein, and this did not stimulate growth of myeloid progenitors in culture. However, the same material significantly enhanced the cloning of IL7-responsive precursors in semisolid agar. The same was true of Ig fusion proteins containing segments of matrix glycoprotein sc1 or the 4-9 clone. Further study will be required to determine if engagement of ligands on pre-B cells by these stromal cell molecules delivers costimulatory signals for survival and differentiation. Alternatively, they might block reception of negative signals that could be present in the FCS or made by neighboring cells. In this context, it is interesting that 4-9 is actually expressed by pre-B cells and could mediate interaction between cells in the same lineage.

Syndecan and biglycan are known to be heparan- or chrondroitin/dermatan–sulfated proteoglycans. These modifications may contribute to cell–cell recognition and could also confer on these molecules the ability to immobilize growth and differentiation factors. Heparin has been shown to block cell adhesion mediated by syndecan (57). Similarly, we found that staining of pre-B cells by our 4-9–Ig, syndecan 4–Ig, and biglycan–Ig fusion proteins was substantially inhibited by addition of heparin (Fig. 5 *A*; unpublished observations). Glycosaminoglycan recognition is a feature of certain other cell adhesion molecules, such as N–cell adhesion molecule (N-CAM) and CD31 (6, 7). Furthermore, this modification is essential for responsiveness of cells to basic FGF (48). Heparan-sulfated proteins can influence the differentiation of hematopoietic cells (36), and this may be in part attributed to their ability to immobilize growth factors such as granulocyte-macrophage colony stimulating factor (G/M-CSF), IL-3, basic FGF, IL7, and interferon-γ (3, 11, 21, 35, 51). All three members of the decorin family, including biglycan, are known to bind TGF-β, but glycosylation is not necessary (13). Osteonectin is not a proteoglycan, but it has been shown to immobilize PGDF (28). It will be important to determine if the stromal cell–derived molecules we isolated bind growth and differentiation factors, as this would suggest another role in the bone marrow microenvironment.

At least 16 names have been used to describe the molecule known as clusterin, and a similarly large number of functions have been ascribed to it (52). It is an abundant and widely distributed protein that is especially rich in tissues undergoing remodeling and where apoptosis is high, but not directly involved in that process (10). Clusterin has a heparin-binding domain and has been shown to cause aggregation of Sertoli cells (for review see 52). The ability of clusterin to bind Ig is of particular interest (63). Maturing pre-B cells make a primitive Ig–receptor complex consisting of μ heavy chains with "surrogate" light chains (Vpre-B and λ_5), and gene targeting revealed this is essential for normal lymphocyte formation (41, 25). It has been speculated that the Ig complex recognizes some ligand on stromal cells (41), and clusterin would seem to be a candidate.

Our findings suggest that 4-9 is a type I transmembrane protein with a functional signal peptide. There is a stretch of 22 hydrophobic residues that might serve as a transmembrane anchor, but this remains to be verified. There is a potential enzyme cleavage site (RH) near this area, suggesting that portions of the molecule might be released as a soluble protein. Another stretch of residues (TRHNHL) in the same region conform to a heparin-binding consensus motif (XBBXBX), where B represents a basic residue and X a hydropathic residue (4). The presumed intracellular portion of 4- 9 has weak sequence homology to myosin heavy chain and related proteins that have a coiled-coil structure.

Northern blot and PCR analyses revealed that the gene is expressed in a number of tissues and in most cell lines. While we have shown that 4-9 can influence the survival and/or proliferation of B cell precursors, it also recognized most mature splenic B cells and a subpopulation of T cells, provided that Mn^{++} was present in the medium. Therefore, the biological function may not be restricted to marrow, and it will be important to learn if it has a role in immune responses. Additional work must also be done to identify the ligand recognized by 4-9. Staining of pre-B cells with the Ig fusion protein was divalent cation–dependent and markedly enhanced by Mn^{++}. It is interesting that the specificity, as well as the affinity, of integrins is influenced by divalent cations (24, 39). One could speculate that while 4-9 expression is relatively broad, its function may be locally controlled in this fashion. However, our preliminary experiments did not positively identify an integrin (VLA4, VLA5, VLA6, or CD11a) as the ligand for 4-9 on pre-B cells. Cadherins, selectins, and other divalent cation–dependent recognition molecules must also be considered.

Expression of extracellular portions of 4-9 in 293T cells as either membrane-anchored or secreted forms had no noticeable influence on morphology or cell growth. However, considerable rounding and detachment of the cells resulted from expression of the full-length molecule. Therefore, normal functions of the native protein in stromal cells may depend on both intracellular and extracellular domains. This dramatic change in morphology of cells that overexpress 4-9 suggests that it may help to orient stromal cells within tissues. It will be important to learn if 4-9 associates with cytoskeletal or other components of the cell, and we provisionally designate it SIM (stromal interaction molecule).

A first application of this new cloning strategy led to the identification of seven extracellular proteins that merit further study as components of lympho-hematopoietic mi-

croenvironments. Ig fusion proteins containing only portions of three such stromal products augmented the responsiveness of pre-B cells to IL7. Native versions of these molecules may be found to participate in adhesive interactions, deliver critical signals, or immobilize and present growth factors to maturing blood cells.

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