A Complex of Glycoproteins Is Associated with V_{preB}/λ_5 Surrogate Light Chain on the Surface of μ Heavy Chain-negative Early Precursor B Cell Lines

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Summary

Monoclonal antibodies (mAbs) have been made specific for the pre-B cell–specific proteins V_{preB} and λ_5 which together form the surrogate light (L) chain. mAbs specific for V_{preB} protein identified the 16-kD molecule associated on precursor B cell lines with λ_5 protein as the product of the V_{preB} gene. Surrogate L chain was detectable even in the absence of μ heavy (H) chain on the surface of early precursor cell lines such as pro-B cell lines where all immunoglobulin (Ig) loci are in the germline configuration, as well as early pre-B cell lines where Ig H chain loci are D_HJ_H rearranged in reading frame I or III, which does not allow the expression of a $D_HJ_HC\mu$ protein. A complex of glycoproteins (200, 130, 105, and 65–35 kD) was identified as coprecipitated with the V_{preb}/λ_5 surrogate L chain in μ H chain–negative precursor B cell lines. The 130-kD protein was most strongly labeled with iodine and most consistently detected in noncovalent association with surrogate L chain. This protein turned out to be a N-linked glycoprotein with a 100-kD protein core and isoelectric point 5.8, indicating that it is distinct from CD43 and the BP-1/6C3 antigen. The surface deposition of the surrogate L chain in association with the newly identified glycoproteins suggests that the surrogate L chain may function as a receptor even before the association with μ H chain in early precursor B cells.

Two genes, V_{preB} and λ_5, are transcribed selectively in precursor B cells (1–3). Mature B cells, Ig-secreting plasma cells, and any other cells in the body so far tested do not express these two genes. The V_{preB} gene has sequence homology to V regions of Ig H and L chain genes, whereas part of the λ_5 gene displays homology to the J and C regions of the Ig λ L chain gene. Therefore, it was expected that the V_{preB} and λ_5 proteins might associate with each other to form an Ig L chainlike structure, the so-called surrogate L chain. It was further expected that the λ_5 protein of surrogate L chain could be disulfide linked via its penultimate cysteine at the COOH-terminal end to μ H chain to form an Ig-like complex. Indeed, the formation of such a μ H chain/λ_5/V_{preB} complex has been proven by gene transfection experiments (4, 5).

Once the λ_5 and V_{preB} genes had been discovered, two proteins called ω and ι were found associated with μ H chains on the surface of some pre-B cell lines (6, 7). One of them (ω) was shown to be the product of the λ_5 gene, and the other (ι) was predicted to be the product of the V_{preB} gene (7). Subsequently, λ_5 protein was detected with polyclonal λ_5-specific antibodies on the surface of pre-B cell lines and bone marrow cells, although it remained to be proven that the 16-kD polypeptide coprecipitated with λ_5 protein was, indeed, the product of the V_{preB} gene (8, 9).

The μ H chain/surrogate L chain complex expressed on pre-B cell lines has been found to transmit biochemical signals to the cells (9, 10), suggesting that this complex could function as a receptor on the surface of pre-B cells. The important role of λ_5 protein in the B cell development was demonstrated by targeted disruption of the λ_5 gene in the germline yielding a λ_5-deficient mutant mouse strain (11). In these mice, B cell development is impaired at the transition from pre-B I to pre-B II cells, resulting in a depletion of 95% of all CD45R(B200)$^+$ B cell precursors in bone marrow and in a delayed appearance of CD5$^-$ B cells in the peripheral lymphoid organs (11, 12).

It has been shown that the 22-kD λ_5 protein and the 16-kD protein (p16)—presumed to be the V_{preB} protein—are expressed together on the surface of μ H chain-negative precursor B cell lines (9). This has raised the questions (*a*) is the p16 identical to V_{preB} protein, and if so; (*b*) how can the V_{preB}/λ_5 surrogate L chain be deposited in the surface membrane in the absence of μ H chain?

In this paper, we describe the generation of mAbs specific for the V_{preB} protein or for λ_5 protein. We use them to study the synthesis, assembly, and surface membrane deposition of V_{preB}/λ_5 surrogate L chain in a panel of precursor B cell lines. We identify molecules that are associated with the V_{preB}/λ_5 surrogate L chain and that might take the place of

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μ H chains on the surface of precursor B cells that do not yet express μ H chains.

Materials and Methods

Cell Lines. The origins and the state of Ig gene loci of pro- and pre-B cell lines used in this study are listed (see Table 1). The cell lines have been described as the following: 63-12 and 63-24 (13); 38B9, 40E1, 204-1-8, 300-19P, 220-8, 28C9, and 204-3-1 (14, 15); 18-81 (16); 70Z/3 (17); NFS5.3 (18); B3-P8-16-1-μ (19); and 38C13 (20). Clone 18, PAL1, and NP were established as described (21, 22). B cell lymphoma cells used as control were IgM (μ, κ)-expressing WEHI231 (23) and IgM (μ, λ)-expressing CH-1 (24). All cell lines were maintained in IMDM supplemented with 10% FCS (Northumbria Biologicals Ltd., Cramlington, UK), 100 U/ml of penicillin-streptomycin (Gibco Laboratories, Grand Island, NY), 2 mM L-glutamine (Gibco Laboratories), and 5×10^{-5} M 2-ME.

Antibodies. Rat mAbs used were M41 (IgG1) specific for murine μ H chain (25), 187.1 (IgG1) specific for murine κ L chain (26), R1-2 (IgG2b) specific for lymphocyte Peyer's patch high endothelial venules (HEV) adhesion molecule 1 [LPAM-1] (murine very late antigen [VLA] 4α) (27), M1/9.3.4 (IgG2a) specific for CD45 (28), and Str10 (IgG) specific for MHC class I (Rolink A., unpublished observations).

Vectors and DNA Transfection. BCMGSHygλ_5 and BCMGSHygV_{preB} were constructed by insertion of λ_5 cDNA (1) or V_{preB} cDNA (3), respectively, into the expression vector BCMGSHyg (4). Ig-nonproducing X63-Ag8.653 myeloma cells (29) were transfected by electroporation with either BCMGSHygλ_5, BCMGSHygV_{preB}, or BCMGSHygλ_5V_{preB} (4) and selected with hygromycin B (0.8 mg/ml, Boehringer Mannheim GmbH, Mannheim, Germany) to obtain single or double transfectants. Transfectants expressing high level of transcripts specific for λ_5 and/or V_{preB} detected by RNA dot blot were subcloned by limiting dilution. The establishment of Ltk$^-$ $\mu\lambda_5V_{preB}$, fibroblast Ltk$^-$ cells transfected with three genes encoding μ H chain, λ_5, and V_{preB}, has been described previously (4).

Preparation of mAbs Specific for λ_5 and V_{preB}. The μ H chain/λ_5/V_{preB} complex was purified by absorption to M41-conjugated Sepharose beads from culture supernatants of a fibroblast transfectant Ltk$^-$ $\mu\lambda_5V_{preB}$ which secreted a soluble form of the complex at a concentration of 200–300 ng/ml (4). Female Lewis rats (Institut für Medizinische Forshung AG, Füllinsdorf, Switzerland) were immunized intraperitoneally four times at 2–8-wk intervals, each time with 7 μg of the complex absorbed to the beads. Sera taken from the animals after a fourth immunization contained antibodies to the complex as detected by immunoprecipitation. One of the animals was given a final boost with intravenous and intraperitoneal injections of 70 μg of the complex eluted from M41-conjugated beads with glycine-HCl buffer (pH 2.7). 3 d after the final injection, spleen cells (3.2×10^8) were fused with murine X63-Ag8.653 myeloma cells (5.7×10^7) using polyethylene glycol 1500 (Boehringer Mannheim) and distributed at a density of 1.6×10^5 spleen cells/well in 1,968 microtiter wells with medium containing HAT and rIL-6 (30). Each well had several colonies of hybridoma in 10 d. Culture supernatants of hybridoma were initially screened by ELISA for reactivity to $\mu\lambda_5V_{preB}$ complex but not to IgM ($\mu\kappa$ and $\mu\lambda$). Supernatants of selected cultures were further characterized by cell surface staining as well as immunoprecipitation and cloned twice by limiting dilution. All the mAbs to λ_5 and V_{preB} described here were of the IgG2a subclass and had κ L chains as determined by the isotyping kit (Serotec, Oxford, UK).

Cell Labeling and Immunoprecipitation. For biosynthetic labeling, cells ($3–5 \times 10^6$) were cultured at 37°C for 4 h in 1 ml of methionine-free RPMI-1640 medium or cysteine-free RPMI-1640 medium (RPMI-1640 Select-Amine kit; Gibco Laboratories) supplemented with 10% dialyzed FCS and 0.3–0.5 mCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham International, Amersham, Bucks, UK) or [^{35}S]cysteine (1,300 Ci/mmole, Amersham) and lysed for 30 min on ice in 1 ml of NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM iodoacetamide, 0.02% NaN_3, 1 mM PMSF, 2 μg/ml aprotinin, and 1 μg/ml pepstatin). In case of Ltk$^-$ $\mu\lambda_5V_{preB}$, cells (2×10^5/0.5 ml/well) were pulsed for 10 h with 0.1 mCi [^{35}S]methionine and culture supernatants were collected.

For cell surface labeling, cells (5×10^7) were suspended in 1 ml PBS and iodinated for 40 min at room temperature by addition of 50 μl 200 mM D-glucose, 1 mCi of $Na^{125}I$ (15.3 mCi $^{125}I/\mu$g of iodine, Amersham), and 50 μl lactoperoxidase (50 U/ml, Sigma Chemical Co., St. Louis, MO)/glucose oxidase (10 U/ml, Sigma Chemical Co.) solution, and lysed as above.

Cell lysates and culture supernatants were precleared with protein A–Sepharose beads (Pharmacia, Uppsala, Sweden) preincubated with normal rabbit serum. Precleared samples were incubated with mAbs at 4°C for 1 h followed by incubation with affinity-purified rabbit anti–rat IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min and then with protein A–Sepharose beads for 1 h. Immunoprecipitates were washed as described (4), suspended in 50 μl of reducing or nonreducing Laemmli sample buffer, boiled for 2 min, and subjected to SDS-PAGE. Gels were stained with Coomassie blue for 20 min, destained for 4 h, soaked for 30 min in Enlightning (New England Nuclear, Boston, MA) only in the case of ^{35}S-labeled samples, and dried. Dried gels were autoradiographed at −70°C on X-Omat AR film (Eastman Kodak, Rochester, NY) with intensifying screens. For deglycosylation of proteins, immunoprecipitates were resuspended in 20 μl elution buffer (0.5% SDS, 50 mM Tris-HCl, pH 8) and boiled for 2 min, followed by addition of 3 μl 10% octylglucoside (Boehringer Mannheim) and 3 μl *N*-glycosidase F (PNGase F[1], 200 U/ml; Boehringer Mannheim) and incubation at 37°C for 18 h. In two-dimensional nonreducing/reducing SDS-PAGE, precipitates were run in the first dimension under nonreducing conditions using a 7–15% gradient polyacrylamide slab gel. The relevant strips were then cut out, incubated in Laemmli sample buffer with 20 mM dithiothreitol for 30 min, and run in the second dimension on a 7–15% gradient SDS-PAGE gel. Two-dimensional IEF/SDS-PAGE and nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE were performed as described (31, 32), using the ampholyte Pharmalyte 2D 3-10 (Pharmacia).

Cell Surface Staining. Cells (10^6) were incubated with 100 μl of hybridoma supernatant diluted 1:1 in staining buffer (PBS, 0.2% BSA, 0.1% NaN_3) for 30 min on ice. After three washes with staining buffer, cells were incubated for 30 min with 100 μl of 1:100 diluted FITC-conjugated MARK1 (mouse mAb to rat κ L chain; Immunotech S.A., Marseille, France). Immunofluorescence was analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Results

Establishment of mAbs Specific for Murine λ_5 and V_{preB}. An Ltk$^-$ fibroblast (Ltk$^-$ $\mu\lambda_5V_{preB}$) transfected with the three

[1] *Abbreviations used in this paper:* NEPHGE, nonequilibrium pH gradient electrophoresis; PNGase F, *N*-glycosidase F.

murine genes coding for μ H chain, λ_5, and V_{preB}, secretes three proteins (86, 22, and 16 kD, respectively) as a complex (4) that can be precipitated with rat mAb M41 specific for mouse μ H chain (Fig. 1 *A*, lane *2*). The complex was purified from culture supernatant of Ltk$^-$ $\mu\lambda_5V_{preB}$ cells by immunoadsorption on M41-conjugated Sepharose beads and used for immunization of rats. Hybridomas were prepared by fusion of spleen cells from an immunized rat. The initial screening of hybridoma supernatants was performed by ELISA using microtiter plates coated with either the $\mu\lambda_5V_{preB}$ complex, IgM ($\mu\kappa$), or IgM ($\mu\lambda$). 11 mAbs were selected which had reactivity to the complex, but not to IgM ($\mu\kappa$ and $\mu\lambda$) (data not shown).

7 out of the 11 mAbs precipitated $\mu\lambda_5V_{preB}$ complex from culture supernatant of the Ltk$^-$ $\mu\lambda_5V_{preB}$ transfectant as did the μ H chain–specific mAb M41 (two representative mAbs are shown in Fig. 1 *A*, lanes *3* and *4*). To identify the reactivity towards antigenic determinants in the complex further, Ig-nonproducing myeloma X63-Ag8.653 cells transfected with either the λ_5 gene or the V_{preB} gene alone, or with the two genes were employed. Five of the mAbs recognized a 22-kD protein present in the λ_5 transfectant but not in the V_{preB} transfectant, whereas two other mAbs detected a 16-kD protein present in the V_{preB} transfectant but not in the λ_5 transfectant (two representatives LM34 and VP245 are shown in Fig. 1 *B*, lanes *2*, *3*, *5*, and *6*). This demonstrates that the former five mAbs are specific for the λ_5 protein, whereas the latter two are specific for the V_{preB} protein. For all subsequent experiments, two mAbs, the λ_5-specific mAb LM34 and the V_{preB}-specific mAb VP245, were used.

λ_5 protein and V_{preB} protein were coprecipitated with each other by either mAb LM34 or VP245 from X63-Ag8.653 cells transfected with both λ_5 and V_{preB} genes (Fig. 1 *B*, lanes *8* and *9*). This shows that λ_5 and V_{preB} proteins can be associated with each other to form a surrogate L chain even in the absence of μ H chain. Thus, the established mAbs recognize λ_5 protein or V_{preB} protein as a single or in a form of V_{preB}/λ_5 surrogate L chain, or in the complex of $\mu\lambda_5V_{preB}$.

The Synthesis and Cell Surface Expression of λ_5 and V_{preB} Proteins in Pro- and Pre- B Cell Lines. The synthesis of the λ_5 and V_{preB} proteins was monitored in a panel of transformed pro- and pre-B cell lines, as well as nontransformed pre-B cell lines, which are thought to represent various stages of B cell development in fetal liver or bone marrow (Table 1). Cells were biosynthetically labeled with [^{35}S]methionine, and cell lysates were analyzed by immunoprecipitation with specific mAbs. All the cell lines tested were found to produce the 22 kD λ_5 and the 16-kD V_{preB} proteins as a complex, irrespective of whether they produced μ H chain or $D_HJ_HC\mu$ protein (33) or none of them (data not shown, but summarized in Table 1).

Next we examined the expression of λ_5 and V_{preB} proteins on the surface of these cell lines by flow cytometry (Fig. 2 and Table 1). All μ H chain–producing pre-B cell lines tested expressed λ_5 and V_{preB} proteins together with μ H chain on their surface (shown for NFS5.3 in Fig. 2, and not shown for others but summarized in Table 1). On the other hand, B cell lines WEHI231 expressing IgM($\mu\kappa$) and CH-1 expressing IgM($\mu\lambda$) were not stained with mAbs LM34 nor VP245 (Fig. 2), showing once more that the mAbs do not crossreact with κ or λ L chains. The two cell lines 38C13 and B3-P8-16-1-μ, which appear to be in transit from pre-B to B cells, expressed on the surface κ L chain as well as λ_5, V_{preB} proteins, and μ H chains (Fig. 2 and Table 1). Proteins

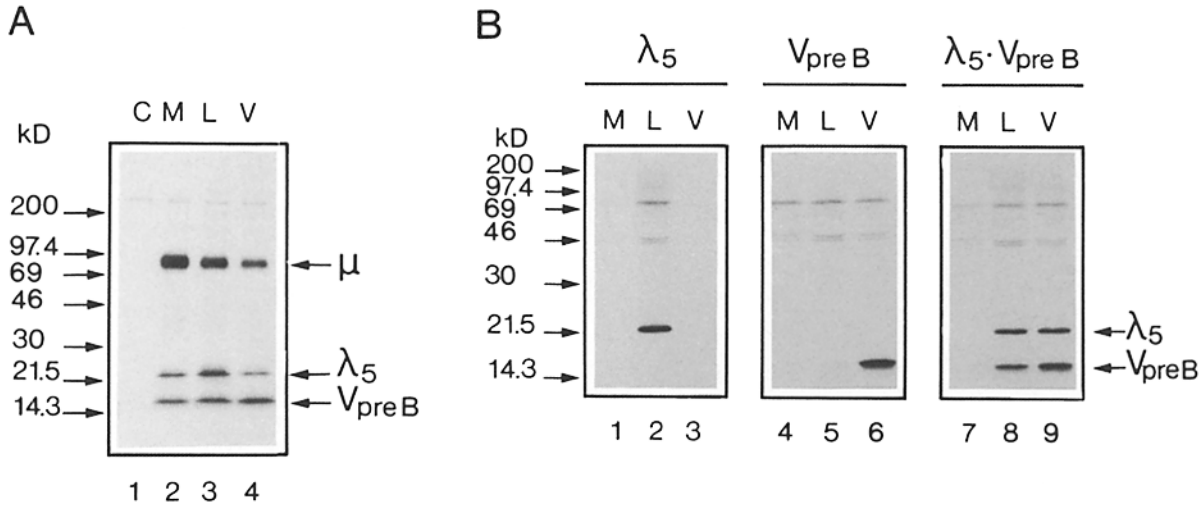

Figure 1. Specificity of mAbs for either λ_5 or V_{preB} protein. (*A*) Culture supernatant (0.1 ml/lane) of Ltk$^-$ $\mu\lambda_5V_{preB}$ transfectant labeled for 10 h with [^{35}S]methionine was reacted with rat mAbs, and immunoprecipitates were analyzed by 4–20% gradient SDS-PAGE under reducing conditions. Gels were soaked in Enlightning, dried, and autoradiographed. Antibodies used include: control mAb ($\gamma_{2a}\kappa$) (C, lane *1*), μ H chain–specific mAb M41 (M, lane *2*), mAb LM34 (L, lane *3*), and mAb VP245 (V, lane *4*). (*B*) X63-Ag8.653 cells were transfected either with λ_5 gene alone, V_{preB} gene alone, or both genes. Each transfectant (4×10^6 cells/ml) was biosynthetically labeled with [^{35}S]methionine for 4 h and lysed with 1% NP-40 lysis buffer. Detergent-soluble lysate was reacted with either mAb M41 (M, lanes *1*, *4*, and *7*), mAb LM34 (L, lanes *2*, *5*, and *8*), or mAb VP245 (V, lanes *3*, *6*, and *9*), and immunoprecipitates were analyzed by 12.5% SDS-PAGE under reducing conditions.

Table 1. *Synthesis and Cell Surface Expression of V_{preB}/λ_5 surrogate L chain in Pro- and Pre-B Cell Lines*

	Origin of cells			Ig gene rearrangements		Synthesis				Surface expression			
Cell line	Mouse	Tissue	Establishment	H	L	λ_5	V_{preB}	μH	L	λ_5	V_{preB}	μH	L
63-12	RAG-2$^{-/-}$	FL	A-MuLV	G	G	+*	+	−	−	+‡	+	−	−
63-24	RAG-2$^{-/-}$	FL	A-MuLV	G	G	+	+	−	−	+	+	−	−
38B9	BALB/c	FL	A-MuLV	DJ/DJ	G	+	+	−	−	+	+	−	−
40E1	BALB/c	FL	A-MuLV	DJ/DJ	G	+	+	−	−	+	+	−	−
Clone 18	BDF_1	FL	Stroma/IL-7	DJ/DJ	G	+	+	−	−	+	+	−	−
PAL1	BDF_1	FL	Stroma/IL-7	DJ/DJ	G	+	+	−	−	+	+	−	−
NP	BALB/c	BM	Stroma/IL-7	DJ/DJ	G	+	+	−	−	+	+	−	−
300-19P	NIH/Swiss	BM	A-MuLV	DJ/DJ	G	+	+	(DJCμ)	−	+	+	(DJCμ)	−
204-1-8	BALB/c	BM	A-MuLV	R	G	+	+	−	−	+	+	−	−
220-8	BALB/c	BM	A-MuLV	VDJ^-/VDJ^-	G	+	+	−	−	+	+	−	−
28C9	BALB/c	FL	A-MuLV	VDJ^+/VDJ^-	G	+	+	+	−	+	+	+	−
204-3-1	BALB/c	BM	A-MuLV	R	G	+	+	+	−	+	+	+	−
18-81	BALB/c	BM	A-MuLV	R	R	+	+	+	−	+	+	+	−
70Z/3	BDF_1	BM	Methyl nitrosourea	VDJ^+/DJ	R	+	+	+	−	+	+	+	−
NFS5.3	NFS/N		Cas-2SM-MuLV	R	R	+	+	+	−	+	+	+	−
B3-P8-16-1-μ	NIH/Swiss	BM	A-MuLV	R	R	+	+	+	κ	+	+	+	κ
38C13	C3H/eB		Dimethylbenzanthracene	VDJ^+/DJ	R	+	+	+	κ	+	+	+	κ

* Proteins were detected by immunoprecipitation of biosynthetically labeled cell lysates with specific mAbs.
‡ Proteins were detected by cell surface staining.
FL, fetal liver; BM, adult bone marrow; G, germline; R, rearranged (DJ, D_HJ_H rearrangement, VDJ^+, productive $V_HD_HJ_H$ rearrangement, VDJ^-, nonproductive $V_HD_HJ_H$ rearrangement).

λ_5 and V_{preB} were also detectable on the surface of the 300-19P cell line which has D_HJ_H rearrangements in both alleles of the IgH locus and produces a $D_HJ_HC\mu$ protein (Fig. 2). The level of expression of λ_5 and V_{preB} was lower compared with other pre-B cell lines that express a full-size μ H chain.

Cell surface expression of λ_5 and V_{preB} proteins was also observed on precursor B cell lines that could not, or did not yet express $D_HJ_HC\mu$ or normal μ H chains. Such cell lines could either be transformed with A-MuLV (38B9 and 40E1 cells) or could be growing on stromal cells in the presence of IL-7 (Clone 18, PAL1, and NP) as shown in Fig. 2 and Table 1. These cell lines have Ig L chain loci in germline configuration and D_HJ_H rearrangements on both alleles of the IgH chain locus, but produce no $D_HJ_HC\mu$ proteins (34). The surface expression of λ_5 and V_{preB} proteins without μ H chain was also detectable on two pro-B cell lines 63-12 and 63-24 derived from the fetal liver of mice in which the RAG-2 gene had been disrupted by targeted integration on both chromosomes (Fig. 2 and Table 1). These cell lines have been shown to have all Ig loci in germline configuration, i.e., are incapable

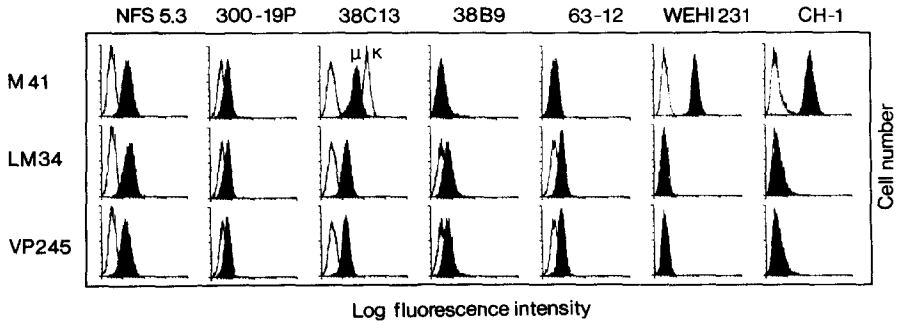

Figure 2. FACS® analysis of V_{preB}/λ_5 surrogate L chains expressed on pro- and pre-B cell lines detected by specific mAbs. Cells were incubated first with either control mAb, μ H chain–specific mAb M41, λ_5-specific mAb LM34, or V_{preB}-specific mAb VP245, and then with FITC-conjugated MARK1. Immunofluorescence was analyzed on a FACScan®. (*Unshaded* histogram) Control staining; (*shaded* histograms) stainings with M41, LM34, and VP245. In the case of 38C13 cells, staining with κ L chain–specific mAb 187.1 is shown as well. NFS5.3, 300-19P, 38B9, and 63-12 cell lines were all negative for surface L chain.

of producing any Ig chain (13). We conclude that the λ_5 and V_{preB} proteins can be deposited on the surface membrane not only in μ H chain–positive precursor B cell lines, but also in progenitor and precursor B cell lines that do not, or do not yet express μ H chains.

Association of λ_5 and V_{preB} Proteins on the Surface of μ H Chain–negative Precursor B Cell Lines. To identify a possible partner(s) of the λ_5 and V_{preB} proteins which might carry them onto the surface of the μ H chain–negative progenitors and precursors, the pro-B cell lines 63-12 and the early pre-B cell line 38B9 were surface labeled with ^{125}I, and the cell lysates were subjected to immunoprecipitation with the specific mAbs (Fig. 3, *A* and *B*, respectively). Whereas no μ H chain was detectable, as expected, the 22-kD λ_5 protein and the 16-kD V_{preB} protein were coprecipitated with each other by either LM34 or VP245 (Fig. 3, *A* and *B*, lanes *3* and *4*). This indicates that λ_5 and V_{preB} proteins are associated with each other to form a surrogate L chain in the absence of μ H chain on the cell surface.

The λ_5 protein in association with μ H chain and V_{preB} protein expressed on the surface of the μ H chain–positive pre-B cell line NFS5.3 has always been found to be poorly iodinated so that it could hardly be seen in autoradiography (Fig. 3 *C*, lanes *2–4*), as was also noted by others (8). In contrast, the λ_5 protein was iodinated fairly well on the μ H chain–negative cell lines (Fig. 3, *A* and *B*, lanes *3* and *4*). This indicates a differential accessibility of tyrosine residues within the λ_5 protein, which is apparently dependent on the presence or absence of μ H chain. This observation is consistent with a recent report by others (9).

The band of 22 kD corresponding to λ_5 protein was not detectable in the nonreducing gels (in Fig. 3 *A*, lanes 7 and *8* and Fig. 3 *B*, lanes *10* and *11*), suggesting that λ_5 protein exists in disulfide-linked complexes on the cell surface. Therefore, the immunoprecipitates with mAb LM34 were further analyzed by two-dimensional SDS-PAGE, in the first dimension under nonreducing conditions, and then in the second dimension under reducing conditions. Monomeric proteins as well as oligomeric proteins not linked by disulfide bonds will migrate identically in both dimensions and can thus be found on the diagonal of gels. In contrast, a disulfide-linked oligomer will migrate as a complex in the first dimension and then will dissociate into separate subunits in the second dimension, therefore appearing as spots below the diagonal. V_{preB} protein was found on the diagonal whereas most of λ_5 protein was detected below the diagonal (Fig. 4, *A*). Some of λ_5 protein was detectable as an off-diagonal spot of 38 kD in first dimension and of 22 kD in second dimension. There was no off-diagonal spot detectable above or below this λ_5 spot. The rest of the λ_5 proteins were found in poorly defined complexes of higher mol wt, making a "streak" rather than distinct spots.

The immunoprecipitates with LM34 were further characterized by two-dimensional NEPHGE/SDS-PAGE analysis (Fig. 5 *A*). The λ_5 protein migrated with a molecular mass of 22 kD and at a fairly basic pH. There was no other spot of 22 kD detectable besides λ_5 protein. These results suggested two possibilities: (*a*) the λ_5 protein in the off-diagonal spot is disulfide-linked to a protein with the same mol wt and the same charge, most likely to itself to form a homodimer; and (*b*) the λ_5 protein is disulfide-linked to another protein that is hardly labeled with ^{125}I. To identify a disulfide-linked partner of λ_5 protein, cells were biosynthetically labeled with [^{35}S]cysteine, and the immunoprecipitates

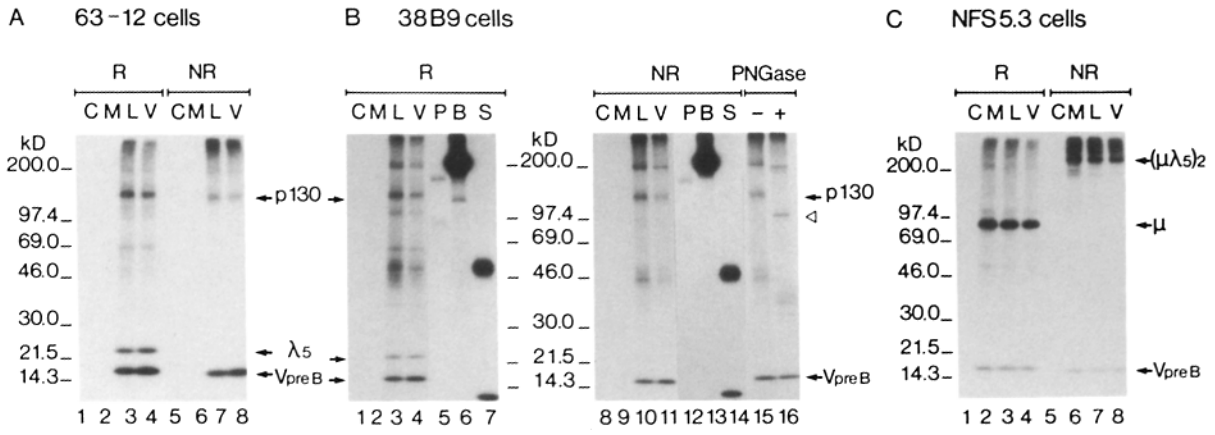

Figure 3. Identification and characterization of molecules associated with V_{preB}/λ_5 surrogate L chain on the cell surface. Cells (*63-12* in *A*, *38B9* in *B*, and *NFS5.3* in *C*) were surface labeled with ^{125}I and lysed with 1% NP-40 lysis buffer. Detergent-soluble lysates (1.5×10^7 cells equivalent/lane in *A* and *B*, and 10^7 cells equivalent/lane in *C*) were reacted with either control mAb (C; lanes *1* and *5* in *A* and *C*, lanes *1* and *8* in *B*), mAb M41 (M; lanes *2* and *6* in *A* and *C*, lanes *2* and *9* in *B*), mAb LM34 (L; lanes *3* and *7* in *A* and *C*, lanes *3* and *10* in *B*), mAb VP245 (V; lanes *4* and *8* in *A* and *C*, lanes *4* and *11* in *B*), LPAM-1 (murine VLA-4α)–specific mAb R1-2 (P; lanes *5* and *12* in *B*), CD45-specific mAb M1/9.3.4 (B; lanes *6* and *13* in *B*), or MHC class I–specific mAb Str10 (S; lanes *7* and *14* in *B*). Immunoprecipitates were analyzed by 7–15% gradient SDS-PAGE under reducing conditions (R; lanes *1–4* in *A* and *C*, lanes *1–7* in *B*) or under nonreducing conditions (NR; lanes *5–8* in *A* and *C*, lanes *8–16* in *B*). In *B*, lanes *15* and *16*, the immunoprecipitates with mAb LM34 were incubated with (lane *16*) or without (lane *15*) PNGase F, followed by SDS-PAGE under nonreducing conditions.

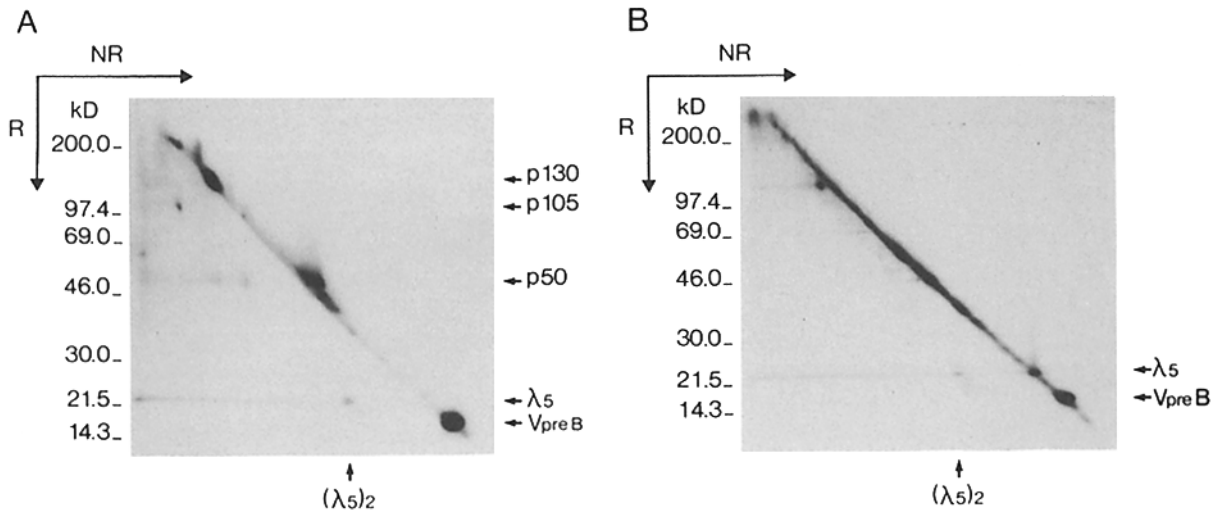

Figure 4. Analysis of the complex with V_{preB}/λ_5 surrogate L chain by nonreducing/reducing two-dimensional SDS-PAGE. The μ H chain–negative pre-B cell line 38B9 was surface labeled with ^{125}I (*A*) or biosynthetically labeled with [^{35}S]cysteine (*B*). NP-40 lysates (3×10^7 cells equivalent in *A* and 3×10^6 cells equivalent in *B*) were reacted with λ_5-specific mAb LM34. Immunoprecipitates were run in the first dimension (*horizontal axis*) under nonreducing conditions on 7–15% gradient SDS-PAGE gel, then in second dimension (*vertical axis*) under reducing conditions on 7–15% gradient SDS-PAGE gel. Immunoprecipitation with V_{preB}-specific mAb VP245 gave comparable pictures (data not shown).

with LM34 were analyzed by two-dimensional nonreducing-reducing SDS-PAGE (Fig. 4 *B*). In contrast to cell surface λ_5 proteins labeled with ^{125}I, the majority of λ_5 proteins labeled with [^{35}S]cysteine were found on the diagonal, indicating that they were not in disulfide-linked complexes. On the other hand, a small fraction of λ_5 proteins was detectable below the diagonal as a spot and a streak in the same position as observed in the ^{125}I-labeled material. There was again no off-diagonal spot detectable above or below the off-diagonal spot of λ_5. This is consistent with the first possibility suggested above, that is, that the λ_5 protein of the off-diagonal spot is a disulfide-linked homodimer. Similar results were obtained when cells were labeled with [^{35}S]methionine (data not shown). Thus, some of the λ_5 protein expressed on the cell surface appears to exist as a disulfide-linked homodimer, whereas most of the λ_5 protein was found in poorly defined complexes with higher mol wt under the condition employed in this study.

Proteins Associated with V_{preB}/λ_5 Surrogate L Chain on the Surface of μ H Chain–negative Precursor B Cell Lines. A complex of proteins of 200, 130, and 105 kD, and several between 65 and 35 kD, was found coprecipitated with V_{preB}/λ_5

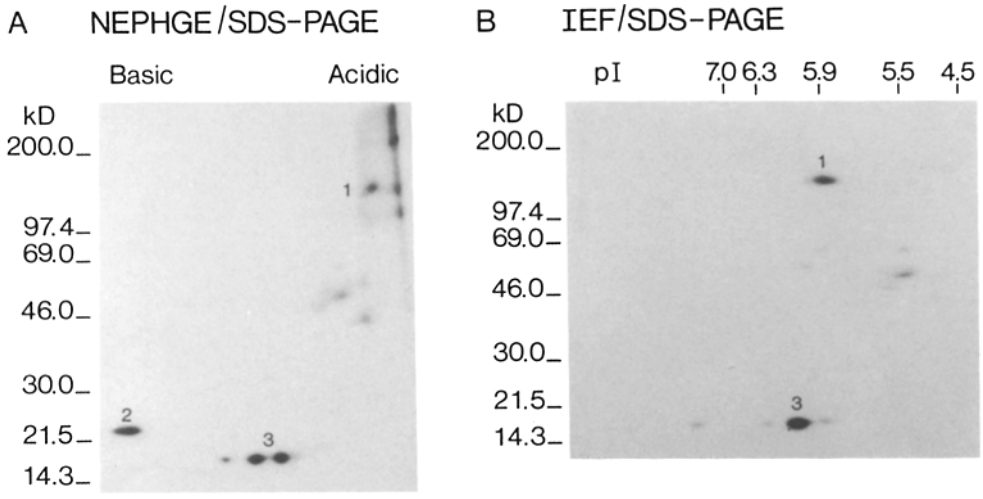

Figure 5. Analysis of the complex with V_{preB}/λ_5 surrogate L chain by two-dimensional NEPHGE/SDS-PAGE and IEF/SDS-PAGE. The μ H chain–negative pre-B cell line 38B9 was surface labeled with ^{125}I, and NP-40 lysates (4×10^7 cells equivalent/precipitation) were reacted with mAb LM34. Immunoprecipitates were separated by charge in the first dimension (*horizontal axis*) in NEPHGE gel (*A*) or in IEF gel (*B*) under reducing conditions, then in second dimension (*vertical axis*) separated by size in 7–15% gradient SDS-PAGE gel under reducing conditions. (*1*) p130; (*2*) λ_5 protein; and (*3*) V_{preB} protein. Note that the λ_5 protein is fairly basic as seen in *A* so that it runs out of the IEF gel under the condition used in *B*.

surrogate L chain from the μ H chain–negative cell lines 63-12 and 38B9 (Fig. 3 *A*, lanes *3*, *4*, 7, and *8*; and Fig. 3 *B*, lanes *3*, *4*, *10*, and *11*). These proteins were not detectable in immunoprecipitates with LPAM-1 (murine VLA-4α)–specific, CD45-specific or MHC class I–specific mAbs (Fig. 3 *B*, lanes *5*–7 and *12–14*). Therefore, the coprecipitation of these proteins is specific for V_{preB}/λ_5 surrogate L chain. The strongly labeled 130-kD protein (p130) was detectable on the diagonal when analyzed by two-dimensional nonreducing–reducing SDS-PAGE (Fig. 4 *A*). This indicates that p130 is associated noncovalently with V_{preB}/λ_5 surrogate L chain on the cell surface. The p130 migrated in pI 5.8 on two-dimensional IEF/SDS-PAGE (Fig. 5 *B*). The p130 was not detectable on the surface of μ H chain–positive NFS5.3 cells as coprecipitated with $\mu\lambda_5V_{preB}$ complex (Fig. 3 *C*, lanes *2–4* and *6–8*).

The intensity in autoradiography of the additional proteins (200, 105, and 65–35 kD) coprecipitated with the surrogate L chain varied in different preparations, compared with that of p130, λ_5, and V_{preB} proteins. It suggests that these molecules may associate loosely with p130 and surrogate L chain. The 200-kD protein and most of the proteins around 35–65 kD were found on the diagonal in the nonreducing/reducing two-dimensional gel (Fig. 4 *A*), indicating that they are bound noncovalently to the $p130/V_{preB}/\lambda_5$ complex. The 50-kD protein migrated faster under nonreducing conditions than under reducing conditions (Fig. 3 *B*, compare lanes *3* and *10*), finding itself above the diagonal on the two-dimensional gel (Fig. 4 *A*). This suggests that the 50-kD protein may contain an intramolecular disulfide bond(s). The 105-kD protein appears to form a disulfide-linked homodimer with an apparent mol mass of 200 kD, since it was detectable as one single off-diagonal spot on the nonreducing/reducing two-dimensional gel (Fig. 4 *A*). Though some materials between 65 and 50 kD appeared to be below the diagonal (Fig. 4 *A*), it remains to be investigated with better methods whether they are disulfide-linked to each other or to the λ_5 protein or to other proteins. In two-dimensional NEPHGE/SDS-PAGE (Fig. 5 *A*) and IEF/SDS-PAGE analysis (Fig. 5 *B*), proteins around 45–65 kD resolved into several spots around pI 5.4–6.0, indicating considerable structural heterogeneity of these surrogate L chain–associated proteins.

Most Proteins Associated with Surrogate L Chain on the Surface of μ H Chain–negative Precursor B Cell Lines Are Glycoprotein. The treatment of the immunoprecipitates with PNGase F, as expected, did not change the apparent mol wt of the V_{preB} and λ_5 proteins. However, it reduced the apparent mol wt of the 200-kD protein (to 190 kD), the p130 (to 100 kD), and the proteins between 45 and 55 kD (to 35–45 kD) as shown in Fig. 3 *B*, lanes *15* and *16*. The two-dimensional nonreducing/reducing SDS-PAGE analysis revealed that the 105-kD subunit of disulfide-linked homodimer reduced its apparent mol wt to 90 kD by treatment with PNGase F (data not shown). We conclude that most of the proteins associated with surrogate L chain on μ H chain–negative precursor B cell lines contain N-linked oligosaccharides and are, therefore, glycoproteins.

Discussion

All μ H chain–producing transformed pre-B cell lines tested so far express the V_{preB}/λ_5 surrogate L chain on the surface. We show that the 16-kD protein, also called ι, is detectable by V_{preB}-specific mAbs. These mAbs, in fact, coprecipitate λ_5 protein and μ H chain. This finally proves that the product of the V_{preB} gene is expressed on the surface of pre-B cells, and that it is the 16-kD protein described previously (8, 9, 35). V_{preB} protein is also detectable on the surface of 300-19P cells in association with $D_HJ_HC\mu$ protein and λ_5. This indicates that the molecular contacts between V_{preB} and λ_5 are strong enough to allow the formation of a trimolecular complex, even though potential contact sites between V_{preB} and V_H are missing.

It is intriguing that V_{preB}/λ_5 surrogate L chains are detectable without μ H chain on the cell surface of pro-B cell lines where all Ig loci are in the germline configuration, as well as on the surface of pre-B cell lines where Ig H chain loci are D_HJ_H rearranged in reading frames that do not allow the expression of $D_HJ_HC\mu$ protein. This indicates that the synthesis of V_{preB}/λ_5 surrogate L chain does not necessarily require the rearrangements of Ig H chain loci. Although this is true for transformed cell lines or for long-term proliferating stromal cell/IL-7–dependent cell lines, it will have to be investigated whether normal precursor cells ex vivo show the same pattern of expression of surrogate L chain.

Misener et al. (9) reported that the complex of λ_5 protein and a 16-kD protein (p16), now identified as V_{preB}, was expressed on the surface of μ H chain–negative A-MuLV–transformed pre-B cell lines carrying two $V_HD_HJ_H$-rearranged alleles. However, in their study, no other molecules were found to be specifically associated with the λ_5/p16 complex (9). Since neither λ_5 nor V_{preB} proteins have typical hydrophobic transmembrane portions, the question arises of how surrogate L chains could be deposited in the surface membrane.

In this study, we have identified some of the candidate proteins that might carry the surrogate L chain onto the surface of pro-B and early pre-B cells. Most consistently detected and most strongly ^{125}I-labeled was a 130-kD protein with pI 5.8 in the protein complex coprecipitated with V_{preB}/λ_5 surrogate L chain from the μ H chain–negative cell lines. It was not detectable in the immunoprecipitates with μ H chain/V_{preB}/λ_5 complex from the μ H chain–positive cell lines. The association of the p130 and the V_{preB}/λ_5 surrogate L chain was found to be noncovalent. The treatment with PNGase F revealed that the p130 is a N-linked glycoprotein with a core protein of around 100 kD. This makes it unlikely that p130 is identical to CD43 (leukosialin), which is expressed at a very early stage of differentiation in the case of B-lineage cells and downregulated upon $V_HD_HJ_H$ rearrangement (36). CD43 is a heavily sialylated O-linked glycoprotein with a protein core of 38 kD and displays an apparent molecular mass of 90–150 kD, depending on cell types. The sequence of the cDNA of murine CD43 predicts only one possible N-linked glycosylation site (37). PNGase F treatment has little if any effect on the size of CD43 (38). In addition, CD43

has no tyrosine residue, so that the protein would hardly be iodinated with lactoperoxidase, whereas the p130 is well iodinated.

p130 is also unlikely to be the BP-1/6C3 aminopeptidase. The BP-1/6C3 antigen, a disulfide-linked homodimer formed by 140-kD N-linked glycoprotein subunits with a protein backbone of 110 kD, also has been shown to be expressed on early B-lineage cells (39, 40). However, the cell surface expression of the BP-1/6C3 and that of p130 do not correlate to each other. The 38B9 pre-B cell line displays p130 but not the BP-1/6C3 on the surface (40). Moreover, p130 is not a disulfide-linked homodimer. Thus, p130 does not appear to be the BP-1/6C3 antigen.

CD22 is a B-lineage–specific N-linked glycoprotein of 130/140 kD in humans and 150 kD in mice with a protein core of 100–110 kD. It has been suggested that CD22 plays a role in B cell–B cell, B cell–monocyte, and B cell–T cell interaction (41–43). We have employed the mAb specific for murine CD22 to examine whether surrogate L chain was coprecipitated with CD22. However, the mAb precipitated a 150-kD protein without V_{preB}/λ_5 surrogate L chain from the 63-12 pro-B cell line, indicating that the p130 is not CD22 (data not shown). It remains to be determined whether the p130 is identical to other known N-linked glycoproteins with a molecular mass of ~130 kD expressed in B-lineage cells, such as CD21 (molecular mass 140 kD with a 120-kD core protein in humans) (41), the IL-6 signal transducer gp 130 (molecular mass 130 kD with a 100-kD core protein) (44), and the common β chain of IL-3R, IL-5R, and GM-CSF-R (45).

Besides p130, several glycoproteins (200, 105, and 65–35 kD) were found coprecipitated with surrogate L chain, even though they appeared to be bound less strongly to surrogate L chain (or to p130). In this study, 1% NP-40 was used to solubilize membrane proteins. It remains to be investigated how different protocols of cell lysis (variations of detergent, salt, pH, etc.) might change the composition of the noncovalently associated protein complex.

When the immunoprecipitates with V_{preB}- or λ_5-specific mAbs were prepared from surface-iodinated μ H chain–negative precursor B cell lines and analyzed by SDS-PAGE under nonreducing conditions, a substantial amount of radioactivity was found at the top of gels (Fig. 3 *A*, lanes 7 and *8*, Fig. 3 *B*, lanes *10* and *11*). Whereas p130 and V_{preB} protein gave distinct bands as observed under reducing condition, λ_5 protein never showed up itself as distinct bands under nonreducing conditions. The nonreducing/reducing two-dimensional SDS-PAGE analysis revealed that some λ_5 protein exists as a disulfide-linked homodimer. However, the majority of λ_5 protein was found in poorly defined complexes with higher mol wt. Moreover, a significant fraction of these complexes was trapped in the first gel and did not enter the second gel of two-dimensional SDS-PAGE. Such "aggregates" were also observed in the $\mu\lambda_5V_{preB}$ complex precipitated from NFS5.3 cells (Fig. 3 *C*, *top*, lanes *6–8*). It has been reported that TCR β chain was expressed in the absence of any other known TCR chains on the surface of immature T cells (46). It is interesting that substantial amounts of the TCR β chain were found in poorly defined high mol wt complexes when the immunoprecipitates were analyzed under nonreducing condition by SDS-PAGE. It needs to be analyzed by better methods which resolve these aggregates of λ_5 protein in order to clarify how many of these complexes are really disulfide bonded and which proteins participate in such covalent bonds. It also remains to be investigated whether these macromolecular forms represent naturally occurring complexes or whether they are artificially generated during preparation of samples.

In this study, we have shown the surface deposition of V_{preB}/λ_5 surrogate L chain in association with the glycoprotein complexes in μ H chain–negative precursor B cell lines. This suggests that the surrogate L chain may function as a receptor on the cell surface even before the association with μ H chain in early precursor B cells. The mAbs established in this study should be useful in elucidating the structure of Ig-like receptor molecules expressed in the early stage of normal B cell differentiation. We are in the process of analyzing the progenitor and precursor B cell compartments in fetal liver and bone marrow of normal and mutant mice to see whether the pattern of surrogate L chain expression and the association with the newly identified partners is the same in normal cells ex vivo. The mAbs may also be useful in our search for a possible ligand(s) of the surrogate L chain complex and in the characterization of developmentally regulated signal transduction in different precursor B cell subpopulations. They should also prove useful in identifying and purifying progenitor and precursor B cells from different lymphoid organs of mice at different times in ontogeny, and in identifying possible defects in B cell development that might arise as a consequence of an abnormal expression of surrogate L chain.

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