

The Proteins Encoded by the V_{preB} and λ_5 Pre-B Cell-specific Genes Can Associate with Each Other and with μ Heavy Chain

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Summary

The murine pre-B cell-specific genes V_{preB} and λ_5 , as well as the murine gene for μ heavy chain, were introduced into Ltk⁻ fibroblast cells which normally do not express these genes. Stable transfectants carrying these genes produced the corresponding proteins of 15.5, 21.5, and 75 kD. They secreted the three proteins as a triple complex that could be immunoprecipitated by μ heavy chain-specific antibodies, consisting of one V_{preB} , one λ_5 , and one μ heavy chain. The μ heavy chain and λ_5 were disulfide-bonded with each other, while the V_{preB} protein was noncovalently associated. These experiments proved that the V_{preB} , λ_5 and μ H chain proteins can form a heavy/light chain-like heterocomplex.

The genes V_{preB} and λ_5 are selectively expressed in pre-B cells (1-3). Because of the homology of V_{preB} to variable (V) regions, and because of the homology of λ_5 to constant regions of λ -light (L) chains, we have proposed that the proteins encoded by the two genes can form a noncovalently bound heterodimer with the general structure of an L chain (4). We have also predicted that λ_5 protein can be covalently disulfide-bonded to μ H chains in pre-B cells because its λ L chain-like constant domain (3) contains the canonical penultimate cysteine residue used in H-L heterodimer binding (1).

To investigate whether the proteins encoded by the μ H chain gene and the V_{preB} and λ_5 genes can, in fact, associate with each other, we now have introduced these genes by transfection into a cell that does not normally express these genes. We have transfected one vector encoding both secreted and membrane forms of the μ H chain together with a bovine papilloma virus-based vector (5) expressing the V_{preB1} and λ_5 cDNAs in the hope of producing a soluble form of $\mu/\lambda_5/V_{preB}$ complex by the transfectants.

Materials and Methods

Plasmid Construction. The expression vector BCMGSNeo was constructed by deletion of a 230-bp EcoRI-XhoI fragment from the 3' end of the murine IL-2 cDNA in vector BCMGNeo-mIL-2 (6) and subsequent self-ligation of the remaining plasmid through NotI linkers, so that BCMGSNeo has XhoI and NotI cloning sites upstream and downstream of cDNA insert, respectively. The XbaI-BamHI fragment of BCMGSNeo was cloned into the corresponding sites of the modified Bluescript plasmid SK (Stratagene, San Diego, CA) to construct SKCMGS. The 800-bp-long XhoI-NotI frag-

ment with the coding sequence of murine V_{preB1} cDNA was isolated from the plasmid SK carrying pZ121 (2) and inserted into the corresponding sites of SKCMGS in place of IL-2 cDNA, resulting in SKCMGS V_{preB} . The 710-bp-long XhoI-NotI fragment with the coding sequence of murine λ_5 cDNA in SK carrying pZ183-1 (1) was cloned into SKCMGS to construct SKCMGS λ_5 in which the enhancer sequence of Ig μ H chain gene has been inserted. The plasmid BCMGSHyg λ_5V_{preB} was prepared from the plasmids SKCMGS V_{preB} , SKCMGS λ_5 , and BCMGSHyg in which the neomycin resistance gene in BCMGSNeo has been replaced by the hygromycin B resistance gene (7), by ligation of the SacII-NheI fragment of each plasmid. The construction of the μ H chain expression vector pR-Sp6 was described previously (8).

Cell Line and DNA Transfection. A murine fibroblast cell line Ltk⁻ was cultured in RPMI 1640 medium supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), 100 U/ml of penicillin-streptomycin (Gibco Laboratories), 2 mM L-glutamine (Gibco Laboratories), and 5×10^{-5} M 2-ME. Ltk⁻ cells were transfected with pR-Sp6 DNA by the protoplast fusion technique as described (5) and selected with 0.5 mg/ml of G418 (Gibco Laboratories). Transfectants expressing high levels of μ H chains were subcloned by limiting dilution and subsequently transfected with BCMGSHyg λ_5V_{preB} followed by selection with 0.5 mg/ml of hygromycin B (Boehringer Mannheim GmbH, Mannheim, FRG).

RNA Dot Blot Analysis. Cytoplasmic RNA was prepared from Ltk⁻ transfectants and transferred to nitrocellulose filters for dot blot analysis as described (9).

Cytoplasmic Staining. Ltk⁻ transfectants were mounted on slide glass by cytospin and fixed with 95% ethanol/5% acetic acid followed by incubation with 100 μ l of 20 μ g/ml FITC-labeled goat anti-mouse μ H chain antibody (Southern Biotechnology Associates, Birmingham, AL) at room temperature for 30 min. Stained cells were examined by fluorescence microscopy.

ELISA. μ H chains in culture supernatants of transfectants were detected by ELISA as described (10).

Immunoprecipitation. Ltk⁻ transfectants were cultured for 10 h at 2×10^5 cells/0.2 ml/well in methionine- and cysteine-free RPMI 1640 medium (RPMI 1640 Select-Amine Kit; Gibco Laboratories) supplemented with 10% dialyzed FCS, and 100 μ Ci of [³⁵S]methionine/[³⁵S]cysteine (1,178 Ci/mmol, Tran³⁵S-label; ICN Biomedicals, Irvine, CA). 100 μ l of ³⁵S-labeled culture supernatant was preabsorbed with 25 μ l of Sepharose beads at 4°C for 1 h, followed by incubation at 4°C overnight with 5 μ l of 1 mg/ml affinity-purified goat anti-mouse μ H chain antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Culture supernatant was then incubated with 25 μ l of protein A-bound Sepharose beads at 4°C for 1 h. After washing three times with NET-NON buffer (650 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% Triton X-100, 0.02% NaN₃, 1 mg/ml ovalbumin) and twice with NET-N buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% Triton X-100, 0.02% NaN₃), protein A beads were suspended in 25 μ l of nonreducing or reducing Laemmli sample buffer and boiled for 2 min. The samples were then subjected to electrophoresis on 6 or 16% of SDS-polyacrylamide gels followed by autoradiography.

Results

Establishment of Cell Lines Transfected with Three Genes Coding for μ H Chain, λ_5 , and V_{preB} . The murine genes encoding μ H chain, λ_5 , and V_{preB} , which are normally expressed in B lineage cells, were introduced successively into fibroblast Ltk⁻ cells that do not express these genes. First, Ltk⁻ cells were transfected with the plasmid pR-Sp6 carrying a functionally rearranged genomic μ H chain gene that encoded both secreted and membrane forms of μ H chains. G418-resistant stable transfectants tested were all positive for μ H chain in cytoplasmic staining. One subclone designated Ltk⁻ μ C37 was selected by limiting dilution and used for further experiments. Ltk⁻ μ C37 cells express μ H chains in cytoplasm at the level comparable to a pre-B cell line NFS5 (11), but not on their cell surface.

The plasmid BCMGSHyg λ_5 V_{preB} was constructed to express both λ_5 and V_{preB} cDNAs in one cell at high levels. This construct has two transcriptional units for expression of inserted cDNA, one for λ_5 and the other for V_{preB} , arrayed side by side with the Ig enhancer sequence in between (Fig. 1). BCMGSHyg λ_5 V_{preB} carries a hygromycin B resistance gene (7) as a dominant selectable marker and 69% of bovine papilloma virus DNA sequence which enables the plasmid to propagate as stable multicopy (30–100 copies/cell)

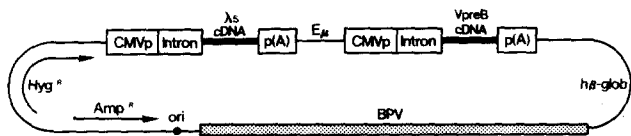


Figure 1. Structure of the expression vector BCMGSHyg λ_5 V_{preB} . The details of its construction are described in Materials and Methods. CMVp, Cytomegalovirus promoter/enhancer; intron, second intron of rabbit β -globin gene; p(A), poly (A) addition and termination signals derived from rabbit β -globin gene; E μ , enhancer of μ heavy chain; BPV, 69% of bovine papilloma virus DNA; h β -glob, part of human β -globin gene; Hyg^R, hygromycin resistance gene; Amp^R, ampicillin resistance gene; ori, pBR322 origin of replication.

extrachromosomal elements in transfected cells (5). This construct was introduced into Ltk⁻ μ C37 cells expressing μ H chain, and stable transfectants were selected with hygromycin B. The RNA dot blot analysis revealed that 63 of 68 transfectants tested expressed substantial amounts of mRNA specific for λ_5 and V_{preB} as well as μ H chains (data not shown).

Ltk⁻ Transfectants Produce and Secrete Heterotrimers Composed of μ H Chain, λ_5 , and V_{preB} . Culture supernatants of Ltk⁻ μ C37 cells transfected with BCMGSHyg λ_5 V_{preB} were screened for the presence of μ H chain by ELISA assay. While untransfected Ltk⁻ cells and Ltk⁻ μ C37 cell did not secrete any detectable level of μ H chain, BCMGSHyg λ_5 V_{preB} -transfected Ltk⁻ μ C37 cells secreted various amounts of μ H chain, which correlated well to the levels of λ_5 - and V_{preB} -specific mRNA expressed in these cells (data not shown). The best transfectant was found to produce and secrete μ H chains at a level comparable to 200 ng/ml of MOPC104E (μ , λ) myeloma protein. ELISA assay with κ and λ light chain-specific antibodies showed that the transfectants did not secrete any light chains.

We next analyzed the structure of molecules secreted from the transfectants to address the question whether secreted molecules are composed of μ H chains alone or together with other peptides such as λ_5 and V_{preB} . Ltk⁻ transfectants secreting μ H chains were biosynthetically labeled with [³⁵S]methionine/cysteine, and culture supernatants were precipitated with μ H chain-specific antibodies followed by electrophoresis on SDS-polyacrylamide gel (Fig. 2). In

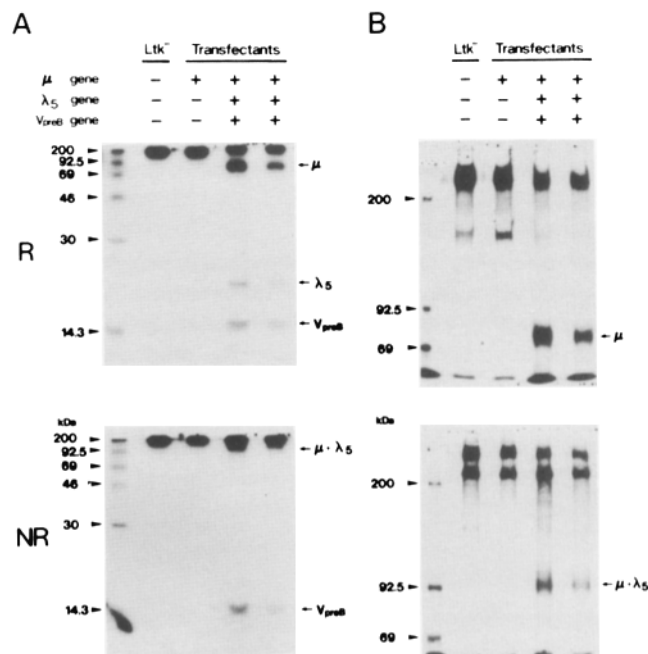


Figure 2. Immunoprecipitation of secreted μ H chains complexed with λ_5 and V_{preB} . Ltk⁻ cells, Ltk⁻ μ C37 cells, and BCMGSHyg λ_5 V_{preB} -transfected Ltk⁻ μ C37 cells were metabolically labeled with [³⁵S]methionine/cysteine, and culture supernatants were precipitated with μ H chain-specific antibodies. Samples were, in reducing (R) or in nonreducing (NR) conditions, subjected to electrophoresis on 16% (A) and 6% (B) of SDS-polyacrylamide gels.

reducing conditions, Ltk⁻ transfectants expressing the three genes gave three specific bands of apparent molecular weights 75,000, 21,500 and 15,500 corresponding to μ H chain, λ_5 and V_{preB}, respectively, because the molecular weights predicted from DNA sequences are 20,000 for λ_5 (3) and 14,000 for V_{preB} (2). In nonreducing conditions, two specific bands of 93,000 and 15,000 mol wt appeared, suggesting that μ H chain, λ_5 and V_{preB} form a heterodimer in which one μ H chain and one λ_5 peptide are disulfide-bonded with each other while one V_{preB} peptide is noncovalently associated.

Discussion

Several polypeptides have been found associated with μ H chains in pre-B cells (4, 12–15). Pillai and Baltimore detected an 18-kD protein called omega, as a candidate for λ_5 , disulfide-bonded to μ H chains in murine pre-B lymphoma cell lines (12, 13), while Kudo et al. (4), Kerr et al. (14), and Hollis et al. (15) found a 22-kD protein as a potential human counterpart. Furthermore, 14-kD (called iota) and 16–18-kD proteins were detected in murine and human pre-B cell lines, respectively (13–15). They were noncovalently associated with μ H chains and putative λ_5 chains, sug-

gesting that they could be candidates for V_{preB}. A peptide-specific antibody against human V_{preB} coprecipitated a ~18-kD protein, a candidate for λ_5 protein and a 70-kD protein, a candidate for μ H chain (4). All these studies suggested that the products of the three genes for μ H chain, λ_5 , and V_{preB} could form a complex. The present study proves that this, in fact, is the case.

The association of μ H chain, λ_5 , V_{preB} appears to be necessary for the secretion of μ H chain from the cell. Transfectants expressing two of the three genes (i.e., μ H and λ_5 , or μ H and V_{preB}) did not secrete μ H chains (unpublished results). Although the μ H chain gene transfected into Ltk⁻ cells encoded both secreted and membrane forms of μ H chain, μ H chains were not expressed on the cell surface (data not shown). This might be expected, since Ltk⁻ cells do not express *mb-1*, a gene encoding a CD3-like 34-kD glycoprotein that has been proposed to anchor μ H chains in the surface membrane (16).

High level expression and secretion of V_{preB}/ λ_5 / μ H chain complex should now enable us to produce better specific antibodies against the native conformation of the complex and to study its three-dimensional structure, and its function in B cell development.

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Note added in proof: After submitting our manuscript, it was brought to our attention that Tsubata and Reth, in a paper also published in this issue of the Journal, show that the transfection of the modified μ H chain gene and the genes coding for λ_5 and V_{preB} into a myeloma cell line leads to the surface expression of μ , λ_5 , and V_{preB} as a complex of proteins associated with each other.

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