Regulation of B Lymphocyte Development by the Truncated Immunoglobulin Heavy Chain Protein D\(\mu\)

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Summary

The development of B lymphocytes from progenitor cells is dependent on the expression of a pre-B cell-specific receptor made up of a \(\mu\) heavy chain associated with the surrogate light chains, immunoglobulin (Ig)\(\alpha\), and Ig\(\alpha\). A variant pre-B cell receptor can be formed in which the \(\mu\) heavy chain is exchanged for a truncated \(\mu\) chain denoted D\(\mu\). To investigate the role of this receptor in the development of B cells, we have generated transgenic mice that express the D\(\mu\) protein in cells of the B lineage. Analysis of these mice reveals that D\(\mu\) expression leads to a partial block in B cell development at the early pre-B cell stage, probably by inhibiting \(V_\mu\) to \(D_{mJH}\) rearrangement. Furthermore, we provide evidence that D\(\mu\) induces V\(_L\) to J\(_L\) rearrangements.

Materials and Methods

Transgenic Constructs. The D\(\mu\) transgenic constructs were created by PCR amplification of D\(_{ml}\) rearrangements, using DNA from large pre-B cells as template. The primers (Fig. 1A, a and b) hybridize to sequences 0.42 kb 5’ of the D\(\mu\) segment and 0.62 kb 3’ of J\(_{m4}\), respectively. The PCR products were sequenced, and a fragment consisting of DFL16.1 joined to J\(_{m4}\) in R F2 was cut with NotI and EcoRI, and cloned into pBluescript. A second construct in which the endogenous promoter was replaced with the mb-1 promoter, was generated by PCR amplification using primers c and b (Fig. 1A), and the fragment was cut with BamHI and EcoRI and cloned into pBluescript containing the mb-1 promoter. The 0.3-kb fragment containing the mb-1 promoter was isolated by PCR according to the published sequence (27). Next, the plasmids were cut with XbaI and XhoI, and ligated with a 9.8-kb Xbal-Xhol fragment from the plasmid p21H22 (10), provided by Dr. T. Leandersson (University of Lund, Lund, Sweden), containing the complete membrane heavy chain.

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During B cell differentiation, the genes encoding the heavy and light chains of the immunoglobulin molecules are assembled from germline gene segments in an ordered fashion (1, 2). Initially, a D\(H\) segment is joined to a J\(H\) segment in the heavy chain locus on both chromosomes. Subsequently, a V\(_H\) gene segment is rearranged to the D\(_{mJH}\) complex. If this renders a functional rearrangement, a \(\mu\) heavy chain is expressed on the cell surface, together with the surrogate light chains encoded by the genes \(\lambda5\) and V\(_{pre-B}\) (3–5). This complex, denoted the pre-B cell receptor (pBCR), has been shown to be of vital importance for maturation of B lymphocytes. Thus, in mice deficient for the surrogate light chains encoded by the genes \(\lambda5\) and V\(_{pre-B}\), the development of B cells is arrested at an early developmental stage due to the absence of a complete \(\mu\) heavy chain.

To investigate the effect of D\(\mu\) expression on B cell differentiation, we generated mice transgenic for the D\(\mu\) protein under control of its endogenous promoter (D\(\mu\)-endo) or, alternatively, under control of the pre-B cell and B cell-specific mb-1 promoter (D\(\mu\)-mb-1; references 25–27).

Abbreviations used in this paper: HSA, heat-stable antigen; pBCR, pre-B cell receptor; RF, reading frame.

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constant region. The plasmids containing the final constructs were digested with NotI and XhoI, the inserts were gel purified and injected into fertilized oocytes of F1(C57BL/6 × CBA) mice. The injected zygotes were transplanted into oviducts of pseudopregnant female mice. Tail DNA from offspring was digested with BamHI, and a 0.3-kb probe comprising the Cμ exon was used for Southern blot, detecting a band of 6.2/6.5 kb in transgenic mice (Fig. 1A). The transgenic band in lane 3 is 0.3 kb shorter than in lane 2, due to the insertion of a BamHI site 3′ of the mb-1 promoter in the construct. (C) Northern blot analysis of total RNA from bone marrow and spleen cells hybridized with a probe complementary to the D13μ exons joint used in the constructs. Blots from transgenic (tg) mice and littermate controls (lm) are shown. Lanes 2, 5, 7, and 6: Dμ-mb-1 line 26. Lanes 3, 4, 7, and 8: Dμ-end line 11. The 1.9-kb Dμ transcript and the 18 S ribosomal RNA are indicated in the figure. GAPDH was used as a control for quantification. (D) Expression of the Dμ protein in transgenic mice. Proteins extracted from bone marrow or spleen cells were analyzed by Western blot. A band of ~69 kD was detected in transgenic mice using an anti-IgM antibody. Blots representing transgenic (tg) mice and littermate controls (lm) are shown. Dμ-mb-1 mice, lanes 1, 2, 5, and 6; Dμ-end mice, lanes 3, 4, 7, and 8. (E) V μ-DJμ and Dμ-Jμ rearrangements in Dμ-end transgenic mice. B220+CD43+ pre-B cells were isolated by cell sorting from transgenic mice (tg) and from littermate controls (lm). Semi-quantitative PCR (28) was performed using a primer hybridizing to a sequence downstream of J2H1, together with primers complementary to either recombination sequences 5′ of all D regions or to members of the J58 family. As the target sequence for the JH primer is not included in the transgenic construct, there is no amplification of the transgene itself. Limited PCR amplification of a non-rearranging locus (λS) was used to normalize the DNA content in the reactions. PCR products were hybridized with a probe complementary to the JH1 and JH2 exons, or to the λS gene. The upper and lower panels show rearrangements to the JH1 and the JH2 gene segments, respectively.

Figure 1. (A) Schematic outline of the transgenic constructs used. The Dμ-end construct included a 0.3-kb fragment containing the endogenous promoter (open box), a DFLH4 rearrangement in RF2, the IgH enhancer (E), and the complete Cμ sequence. Arrowheads, the location of PCR primers used to generate the constructs. The probe used for Southern blot analysis is depicted in the figure together with the size of the BamHI fragment detected in transgenic mice (6.5 kb). The construct used to create Dμ-mb-1 transgenic mice was derived from the Dμ-end construct by replacing the region upstream of the ATG with a 0.3-kb fragment containing the mb-1 promoter (shaded box). B, BamHI; N, NotI; R, EcoRI; Xb, XbaI; Xhol. (B) Northern blot analysis of genomic tail DNA digested with BamHI and hybridized with Cμ exon 1 as a probe. The 6.5-kb Dμ transgenic band and the ~10-kb band representing the endogenous Cμ locus are indicated. Lane 1, C57/BL6; lane 2, Dμ-end, founder 13; lane 3, Dμ-mb-1, founder 23. The transgenic band in lane 3 is 0.3 kb shorter than in lane 2, due to the insertion of a BamHI site 3′ of the mb-1 promoter in the construct.
isolated using Ultraspec RNA isolation system (Biotecx, Houston, TX). The RNA was electrophoresed in a 1.2% agarose/formaldehyde gel, transferred to Zeta-Probe GT blotting membranes (BioRad), and hybridized according to the manufacturer’s recommendations. A 0.8-kb fragment from the transgene construct, spanning the DHJH complex, was used as a probe to detect D<sub>m</sub>transcripts. k transscripts were detected using a 0.4-kb fragment comprising the 3' portion of the C<sub>k</sub> gene, and a 0.9-kb probe containing part of the mouse mb-1 gene (25; provided by Dr. Michael Reth, Institute for Biology III, Freiburg, Germany) was used to determine the amount of B cell–derived RNA in the samples.

Western Blot Analysis. Proteins were prepared by lysing 3–10 × 10<sup>6</sup> cells from bone marrow, or from spleen, in 100 μL sample buffer (135 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 10% 2-mercaptoethanol, and bromophenolblue indicator [BFB]) and applying the suspension to a 5–15% SDS-PAGE gradient gel. Fractionated proteins were electroblotted onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) using a Trans-Blot cell (BioRad). Immunodetection of the D<sub>m</sub> chain was carried out using a horseradish peroxidase–labeled anti-IgM antibody (Southern Biotechnology Associates, Birmingham, AL) and an ECL Western blotting kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer’s protocol.

Flow Cytometry Analysis and Cell Sorting. Bone marrow cells were flushed out of the femurs with HBSS. Spleen cells were obtained by homogenization of the organ in the same medium. Cells were collected by centrifugation, resuspended in FACS medium (3% fetal calf serum and 0.1% sodium azide in PBS), counted, and 10<sup>6</sup> cells/25 μL were incubated with antibodies. The antibodies used were: biotin-coupled anti-B220 RA3.6.B2 (30), FITC-labeled anti-IgM (Southern Biotechnology Associates), FITC-labeled anti-CD43 (PharMingen, San Diego, CA), biotin-coupled anti–heat stable antigen (HSA) (PharMingen), and streptavidin PE–labeled anti–BP-1 (PharMingen). PE– and Cy-chrome–conjugated streptavidin were obtained from PharMingen. Stained cells were analyzed on a FACScalibur<sup>®</sup> (Becton Dickinson, Mountain View, CA). For cell sorting, bone marrow cells were stained with the same reagents and separated on a FACStar Plus<sup>®</sup> (Becton Dickinson).

### Table 1. Number of IgM<sup>+</sup> B Cells and of Total Nucleated Cells

| Transgenic construct | Founder | TG/LM * | Newborn liver | 3-wk-old spleen | Adult spleen |
|----------------------|---------|---------|---------------|----------------|-------------|
|                      |         |         | Nucleated cells | IgM<sup>+</sup> cells | Nucleated cells | IgM<sup>+</sup> cells |
|                      |         |         | × 10<sup>6</sup> | × 10<sup>4</sup> | n | × 10<sup>6</sup> | × 10<sup>4</sup> | n | × 10<sup>6</sup> | × 10<sup>4</sup> | n |
| D<sub>m</sub>-endo 11 | TG      | 12.5 (± 3.9)<sup>†</sup> | 3.5 (± 0.6) | 3 | 18.2 (± 6.3) | 2.9 (± 2.6) | 4 | 37.0 (± 4.2) | 18.2 (± 1.9) | 2 |
| LM                   | 16.4 (± 2.4) | 51.5 (± 9.8) | 4 | 36.2 (± 6.2) | 13.7 (± 4.1) | 5 | 79.0 (± 19.8) | 42.8 (± 7.1) | 2 |
| D<sub>m</sub>-mb-1 26 | TG      | 7.1 (± 1.4) | 7.4 (± 2.2) | 5 | ND | ND | 5 | ND | ND | ND |
| LM                   | 8.2 (± 2.3) | 22.3 (± 8.9) | 5 | ND | ND | 5 | ND | ND | ND |

*TG, transgenic; LM, littermates.
†Numbers given within parenthesis represent standard deviation.

**Figure 2.** Flow cytometric analysis of newborn liver and bone marrow cells from D<sub>m</sub>-endo transgenic mice, D<sub>m</sub>-mb-1 transgenic mice, and littermate controls. Cells were stained with anti-B220-PE and anti-IgM-FITC and analyzed on FACScan<sup>®</sup>. The lymphocyte population was gated according to standard forward-and side-scatter values. The numbers above the framed areas represent the percentage of B220<sup>+</sup>IgM<sup>-</sup> and B220<sup>+</sup>IgM<sup>+</sup> cells out of the total number of gated lymphocytes.
Results and Discussion

D\(\mu\) Expression Leads to Arrest in V\(\mu\) to D\(\mu\)\mbox{-}J\(\mu\) Rearrangements. Five founder transgenic mouse lines were established expressing D\(\mu\), under the control of the endogenous D\(\mu\) promoter, and four lines were established with D\(\mu\) expression controlled by the mb-1 promoter (25–27; Fig. 1 A). Each of the founder mice were crossed to C57BL/6 mice and were analyzed for integration of the transgene by genomic Southern blots using a probe hybridizing to the C\(\mu\)I exon (Fig. 1 B). Transcription of transgenic D\(\mu\) in splenic and bone marrow cells from transgenic mice was demonstrated by Northern blot analysis (Fig. 1 C). Western blot analysis of cell lysates was used to confirm the expression of transgenic D\(\mu\) protein. Thus, D\(\mu\) protein was readily detected in bone marrow cells and in spleen cells from transgenic, but not from littermate mice (Fig. 1 D).

To directly test if the expression of D\(\mu\) protein would affect the V\(\mu\)D\(\mu\)H\(\mu\) rearrangement process, the relative amount of complete V\(\mu\)D\(\mu\)H\(\mu\) and of incomplete D\(\mu\)H\(\mu\) rearrangements was estimated in B220\(^{+}\)CD43\(^{+}\) early B cell progenitors using a semiquantitative PCR assay (28). As illustrated in Fig. 1 E, the relative amount of complete V\(\mu\)H\(\mu\) D\(\mu\)H\(\mu\) rearrangements was found to be severely reduced in the transgenic mice compared to littermate controls. In contrast, D\(\mu\)H\(\mu\) rearrangements were more abundant in the transgenic mice. Together, these data provide evidence for that expression of the D\(\mu\) protein mediates inhibition of V\(\mu\)H\(\mu\) to D\(\mu\)H\(\mu\) rearrangements.

Partial B Cell Depletion in D\(\mu\) Transgenic Mice. To study the effect of the transgenically expressed D\(\mu\) protein on the B cell compartment, newborn liver, bone marrow, and spleen cells from two transgenic lines were analyzed by flow cytometry. Analysis of B lymphocytes from newborn mice revealed an \(~3\)- and 15-fold reduction of IgM positive cells in the liver (Table 1, Fig. 2). In 3 wk-old mice, B cell numbers were reduced about fourfold in transgenic mice compared to littermate controls (Table 1). In adult spleen, the number of total B cells was approximately two-fold lower in the transgenic mice (Table 1), whereas the T cells numbers were apparently unchanged (data not shown). At all time points analyzed, no significant difference in the proportion of CD5\(^{+}\) and CD5\(^{-}\) B cells in the peritoneum was observed, indicating that the generation of B-1 cells and of conventional B cells were similarly affected by the transgenic D\(\mu\) expression (data not shown).

Impairment of B Cell Differentiation in D\(\mu\) Transgenic Mice Occurs at the Pro/Pre B Cell Stage. To identify the stage at which the B cell development was affected by the transgenic expression of D\(\mu\), bone marrow cells from adult mice were analyzed by flow cytometry. The number of immature and mature B cells (B220\(^{+}\)IgM\(^{+}\)) was diminished approximately two- to threefold in transgenic mice, whereas the B220\(^{+}\)IgM\(^{-}\) population, including most B cell progenitors, was similar or only slightly reduced compared to littermate controls (Fig. 2).

The stages of B cell differentiation have been subdivided into fractions (A–F) on the basis of expression of the cell surface markers B220, CD43, HSA, BP-1, and IgM (31). Analysis of bone marrow cells using these markers revealed that the B220\(^{+}\)CD43\(^{+}\) early progenitors were slightly increased in transgenic mice, whereas B220\(^{-}\)CD43\(^{+}\) cells were fourfold reduced compared to littermate controls (Fig. 3). These results suggested that the observed block in B cell development induced by D\(\mu\) expression occurs before the transition of late pro-B cells to the pre-B cell stage.

To further dissect at what point in early B cell differentiation D\(\mu\) expression exerts its effect, BP-1, CD43, and HSA expression was used to subdivide B cell progenitor cell populations (31). As shown in Fig. 4, the CD43\(^{+}\)BP1\(^{+}\) cell population (late pro-B cells) was slightly increased in transgenic mice compared to littermate controls. However, although the number of CD43\(^{+}\)BP1\(^{+}\) cells with low expression of HSA was almost twofold higher in the transgenic versus wild-type mice, the number of CD43\(^{+}\)BP1\(^{+}\) cells with high level expression of HSA was similar. Thus,
the partial block in B cell development induced in the D\textmu transgenic mice occurs at the transition of the fraction C to the fraction C' in the nomenclature of Hardy et al. (31), i.e., at the developmental stage where \textmu-chain expression on the cell surface is required for further differentiation. Our results provide support for the hypothesis that the D\textmu pBCR can mediate a block in B cell development, probably by inhibiting further \textit{VH} to \textit{DHJH} rearrangements (32, 33) similar to the pBCR (10–13). This mechanism appears, however, to allow leakage of cells that can complete \textit{VHDHJH} rearrangement in the presence of D\textmu expression. This is not surprising in view of the observed production of endogenous rearrangements in transgenic mice expressing an IgH chain (10–13). It is predicted from the proposed action of D\textmu expression that most or all of these mature B cells should contain only one complete \textit{VHDHJH} rearrangement. Experiments addressing this issue are presently ongoing.

D\textmu Induces Light Chain Rearrangements. In addition to inhibiting \textit{VH} to \textit{DHJH} rearrangements, D\textmu expression has been suggested to mediate induction of \textit{VL} to \textit{JL} rearrangements (21, 32). If so, progenitor B cells of the D\textmu transgenic mice would be expected to rearrange the light chain locus despite the arrest in B cell development and the possible impairment of \textit{VH} to \textit{DHJH} rearrangements. To test this hypothesis, we analyzed the levels of expression of k-chain mRNA in bone marrow cells. The levels of k transcripts were found to be similar in transgenic mice compared to littermate controls (Fig. 5). Since in transgenic mice there is a three- to four-fold reduction in the B cell progenitors that normally produce L chain transcripts, these results suggest that D\textmu expression induces \textit{VL} to \textit{JL} rearrangements in progenitors that normally would contain the light chain loci in germline configuration. It appears, thus, that D\textmu can replace the complete \textmuH chain in terms of mediating induction of \textit{VL} to \textit{JL} rearrangements.

We conclude from these results that the D\textmu pBCR can mediate a block in B cell development, probably by inhibiting \textit{VH} to \textit{DHJH} rearrangements, as well as inducing \textit{VL} to \textit{JL} rearrangements. In contrast, D\textmu cannot substitute for the requirement of \textmuH chain expression for pre-B cell transition. These observations are in agreement with previous reports demonstrating that D\textmu counterselection is mediated through the transmembrane domain of the membrane D\textmu protein (33) and is dependent on the expression of Ig\beta (23) and Syk (22). It has been suggested that the inability of D\textmu to mediate pre-B cell transition would be due to a failure to pair with L chains (24). This explanation seems unlikely, however, because counterselection of the D\textmu protein-encoding RF2 appears to occur before the stage of L chain expression (34). An alternative explanation may be that the D\textmu pBCR and the pBCR generate qualitatively different signals (12). Further analysis of the D\textmu transgenic mice will be able to directly assess this possibility.

We thank Drs. A. Cumano, J. Demengeot, B. Eriksson, and P. Perreira for discussions and for reviewing the manuscript.
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