Lineage Commitment in the Thymus: Only the Most Differentiated ($TCR^{hi}bcl\text{-}2^{hi}$) Subset of $CD4^+CD8^+$ Thymocytes Has Selectively Terminated CD4 or CD8 Synthesis

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Summary

Lineage commitment is a developmental process by which individual $CD4^+CD8^+$ (double positive, DP) thymocytes make a decision to differentiate into either $CD4^+$ or $CD8^+$ T cells. However, the molecular event(s) that defines lineage commitment is controversial. We have previously proposed that lineage commitment in DP thymocytes can be molecularly defined as the selective termination of CD4 or CD8 coreceptor synthesis. The present study supports such a molecular definition by showing that termination of either CD4 or CD8 synthesis is a highly regulated event that is only evident within the most differentiated DP subset ($CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$). In fact, essentially all cells within this DP subset actively synthesize only one coreceptor molecule. In addition, the present results identify three distinct subpopulations of DP thymocytes that define the developmental progression of the lineage commitment process and demonstrate that lineage commitment is coincident with upregulation of TCR and bcl-2. Thus, this study supports a molecular definition of lineage commitment and uniquely identifies $TCR^{hi}bcl\text{-}2^{hi}$ DP thymocytes as cells that are already committed to either the CD4 or CD8 T cell lineage.

Thymocytes develop through a series of stages which can be distinguished by variations in surface expression of the coreceptor molecules CD4 and CD8 (reviewed in reference 1). Double-negative (DN)[1] cells expressing neither CD4 nor CD8 ($CD4^-CD8^-$) mature into double-positive (DP) cells expressing both CD4 and CD8 ($CD4^+CD8^+$) which, in turn, develop into single-positive (SP) cells that selectively express CD4 ($CD4^+CD8^-$) or CD8 ($CD4^-CD8^+$). Development of immature DP thymocytes into mature SP thymocytes is a highly regulated process in which only those DP thymocytes with TCR of appropriate specificities are positively selected to further differentiate into SP T cells. The process of positive selection (reviewed in reference 2) involves at least three major cellular events: (*a*) conversion of a DP thymocyte expressing both CD4 and CD8 coreceptor molecules into a SP T cell expressing only one coreceptor molecule, referred to as lineage commitment; (*b*) conversion of a short-lived DP thymocyte into a long-lived SP T cell; and (*c*) conversion of a functionally incompetent DP thymocyte into a functionally competent SP T cell. The cellular and molecular bases for these processes remain largely a matter of speculation. In this study we have addressed the first issue of lineage commitment.

The cellular and molecular signals that induce lineage commitment in DP thymocytes remain controversial (reviewed in reference 3). Studies to identify such signals have not focused directly on DP thymocytes in which lineage commitment signals are generated but rather have assessed lineage commitment either by the successful generation of SP T cells or by the appearance of populations that appear to be in transition to a SP phenotype (4–13). Indeed, lineage commitment for T cells is best defined developmentally as an irreversible commitment to differentiate into either a $CD4^+$ or $CD8^+$ T cell. Unfortunately, such a developmental definition does not permit lineage commitment to be recognized in signaled DP thymocytes when it occurs, but only after it has resulted in its successful differentiation into a SP T cell. Consequently, we have formulated a molecular definition of lineage commitment that can be applied directly to DP thymocytes. We have proposed that lineage commitment be molecularly defined as the selective termination of synthesis of one coreceptor protein in a cell that expresses both coreceptor proteins on its surface (12). That a DP thymocyte expresses both CD4

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; SP, single positive.

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and CD8 coreceptor proteins on its surface is definitive evidence that it was recently synthesizing both coreceptor molecules, and whether or not it continues to actively synthesize both coreceptor molecules we think reflects its lineage commitment status.

We have recently described an assay, the coreceptor reexpression assay, which assesses individual DP thymocytes for coreceptor synthesis (12). By our molecular definition of lineage commitment, DP thymocytes synthesizing both CD4 and CD8 coreceptors are uncommitted cells, whereas DP thymocytes synthesizing only CD4 or CD8 coreceptor molecules are lineage committed cells. However, it has been argued that selective termination of synthesis of one coreceptor protein may occur sporadically in DP thymocytes, at random developmental points, and not reflect a developmentally regulated lineage commitment event.

We now report that selective termination of CD4 or CD8 coreceptor synthesis does not occur capriciously in DP thymocytes, but is developmentally restricted to the most differentiated subpopulation of DP thymocytes which is characterized as $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$. In fact, all $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$ DP thymocytes have terminated synthesis of one coreceptor molecule despite their surface expression of both CD4 and CD8 proteins. As a result, it is the immediate precursors of these cells, i.e., $CD5^{hi}CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes, that appear to be coordinately signaled in the thymus to terminate synthesis of one coreceptor molecule and to upregulate both TCR and bcl-2 expression. Thus, the present study identifies the phenotype of cells within the DP population that have undergone lineage commitment and shows that lineage commitment is coincident with high levels of TCR and bcl-2 expression.

Materials and Methods

Mice and Thymocyte Preparations. C57BL/6 (B6) and $TCR\alpha^{-/-}$ mice (14) were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed and bred in a specific pathogen-free facility. Thymuses were dissected from mice between 6 and 10 wk of age and single cell suspensions were made by gently teasing dissected lobes with forceps and filtering over nylon.

Staining and Sorting. Thymocytes were stained and sorted as previously described (12). In brief, 5×10^5 cells were distributed in wells of a 96-well plate (Nunclon, Roskike, Denmark), pelleted, and stained for 30 min at 4°C with saturating concentrations of PE-conjugated anti-CD4 mAb (GK1.5; Becton Dickinson & Co., Mountain View, CA), FITC-conjugated anti-CD8 mAb (53-6-7; Becton Dickinson), and, where indicated, biotinylated anti-CD5 mAb (53.7.3; Becton Dickinson & Co.), biotinylated anti-CD69 mAb (H1.2F3; PharMingen, San Diego, CA) or biotinylated anti-TCRβ mAb (H57-597; PharMingen) in 30 μl final volume of staining medium (0.5% BSA, 0.5% NaN_3 in HBSS). PE-conjugated, FITC-conjugated, and biotinylated anti-human CD3 mAb (Leu 4; Becton Dickinson & Co.) were used as controls for nonspecific staining. All staining was also performed in the presence of anti-FcReceptor (FcR) antibody (2.4G2; Becton Dickinson & Co.) to block nonspecific antibody binding to FcRs. Cells were washed twice with 150 μl of staining medium and if a second step were required, were stained again in 30 μl final volume of saturating concentrations of either Texas-red streptavidin or RED670-streptavidin (GIBCO BRL, Gaithersburg, MD) for 5 min at 4°C. Cells were washed twice again and analyzed by flow cytometry. For sorting, cells were stained at a final concentration of 10^8/ml in 12 × 75 mm tubes (no. 2058; FALCON, Becton Dickinson & Co.) using saturating amounts of PE-conjugated anti-CD4 mAb, FITC-conjugated anti-CD8 mAb and biotinylated anti-CD5 mAb. After washing two times with staining medium, cells were resuspended at a concentration of 10^8/ml and stained with saturating concentrations of Texas red (TR)-streptavidin (GIBCO BRL). After sorting, pronase treatment, and culture cells were typically restained with the same antibody combinations for analysis. In those cases when it was necessary to stain for an additional marker, we restained with PE anti-CD4 and FITC anti-CD8 as usual, but used distinct biotinylated antibodies (biotinylated anti-TCRβ mAb (H57-597, PharMingen) or anti-murine bcl-2 mAb (3F11, PharMingen) followed by a biotinylated anti-hamster mAb (G94-56, PharMingen) in combination with RED670-conjugated streptavidin to avoid confusion with any residual anti-CD5/TR-streptavidin conjugates. Staining with anti-bcl-2 required that the cells be permeabilized by treatment with 0.03% saponin and we followed the method described by Veis et al. (15). Flow cytometry was performed on a Becton Dickinson FACStar® Plus and analyzed using software designed by the Division of Computer Research and Technology at the National Institutes of Health except in cases where RED-670 was used as a second step, when cells were analyzed on a Becton Dickinson FACScan® with Cell Quest software. Dead cells were excluded electronically by gating on forward scatter and propidium iodide intensity when analyzed on the FACStar® Plus and by gating on forward and side scatter when analyzed on the FACScan®.

Pronase Treatment. The coreceptor reexpression assay was performed as previously described (12). In brief, sorted cells were washed two times with PBS and resuspended at 1–5 × 10^6/ml with or without 0.04% Pronase (Calbiochem Novabiochem, La Jolla, CA) in PBS. They were incubated for 15 min at 37°C and then pelleted and incubated with fresh pronase for another 10 min at 37°C. Cells were subsequently washed three times with complete medium then distributed in 0.5-ml vol into 24-well plates at a final concentration of 0.5–2.0 ×10^6 per ml. Cells were incubated overnight (12–16 h) at 4°C or 37°C, stained, and analyzed.

Results

Selective Termination of Either CD4 or CD8 Coreceptor Synthesis in Defined Subpopulations of DP Thymocytes. Because CD4 and CD8 surface proteins can survive for hours on the surface of DP thymocytes, DP thymocytes can selectively terminate synthesis of one or the other coreceptor molecule without an obvious change in surface phenotype. To detect the coreceptor molecules that DP thymocytes are actively synthesizing, we have utilized the coreceptor reexpression assay (Fig. 1). In brief, thymocytes are stripped of surface CD4 and CD8 proteins by extracellular treatment with low concentrations of the protease pronase, washed, and then cultured overnight at 37°C in medium alone to allow surface reexpression. Surface reexpression of coreceptor molecules in this assay has previously been shown to require new transcription and active protein synthesis and so provides a convenient demonstration of the

coreceptor molecules that individual DP thymocytes are actively synthesizing (12). In the present study we utilized the coreceptor reexpression assay to determine if any relationship existed between the selective termination of coreceptor synthesis and developmental maturity. As well-characterized markers of developmental maturity we utilized surface expression of CD5 and CD69 (9, 16–21).

Surface expression of CD5 and CD69 is markedly heterogeneous among DP thymocytes, with DP thymocytes contained within cell populations expressing low, intermediate, and high levels of each marker (Fig. 2, *a* and *b*). More importantly, surface CD5 and CD69 expression is directly correlated with developmental maturity such that the least differentiated DP thymocytes express low amounts of CD5 and CD69 and the most differentiated DP thymocytes express high levels of CD5 and CD69 (17, 19, 20). Consequently, to determine if termination of coreceptor synthesis occurred in a developmentally restricted subpopulation of DP thymocytes, we performed the coreceptor reexpression assay on thymocytes that were electronically purified by 3-color cell sorting into DP thymocytes expressing low/intermediate versus high levels of surface CD5 and CD69 (Fig. 3, *a* and *b*). All DP thymocytes expressing low/intermediate levels of CD5 or CD69 either synthesized both coreceptor molecules or synthesized no coreceptor molecule (Fig. 3, *a* and *b*, *upper panels*); essentially none of these cells selectively synthesized only one coreceptor molecule (Fig. 3, *a* and *b*). In contrast, DP thymocytes expressing high levels of surface CD5 or CD69 did contain cells that were selectively synthesizing only one coreceptor molecule (Fig. 3, *a* and *b*, *lower panels*). DP thymocytes selectively synthesizing CD8 usually outnumbered thymocytes selectively synthesizing CD4. We suspect this is because thymocytes that have selectively terminated coreceptor synthesis lose surface CD8 expression more quickly than surface CD4 expression, a possibility consistent with observations by other investigators (22, 23). We also observed a

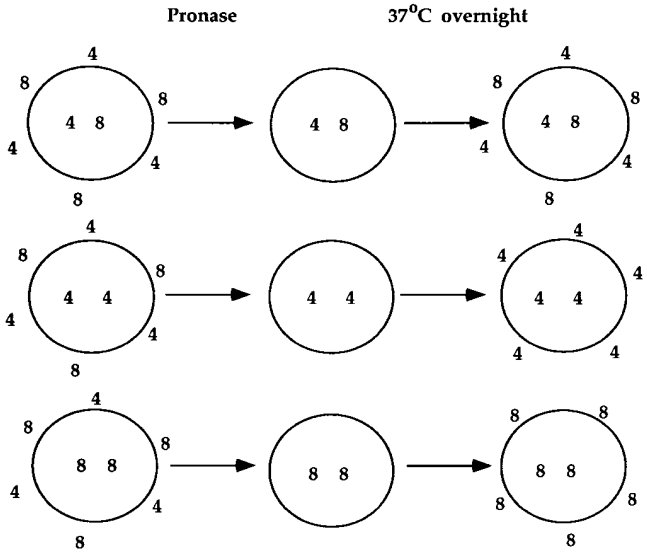

Figure 1. Coreceptor reexpression assay. Thymocytes that express both CD4 and CD8 on their surfaces may no longer be actively synthesizing both CD4 and CD8 coreceptor molecules. To reveal the coreceptor(s) that individual $CD4^+CD8^+$ thymocytes are actively synthesizing, thymocytes are treated with low concentrations of the protease pronase which strips the surface of the cell of existing CD4 and CD8 protein molecules. Cells are then allowed to reexpress the coreceptor proteins they are actively synthesizing during overnight culture at 37°C. In the schematic, surface CD4 and CD8 proteins are indicated by 4 or 8 outside the circle, and internal CD4 and CD8 mRNAs are indicated by 4 or 8 inside the circle.

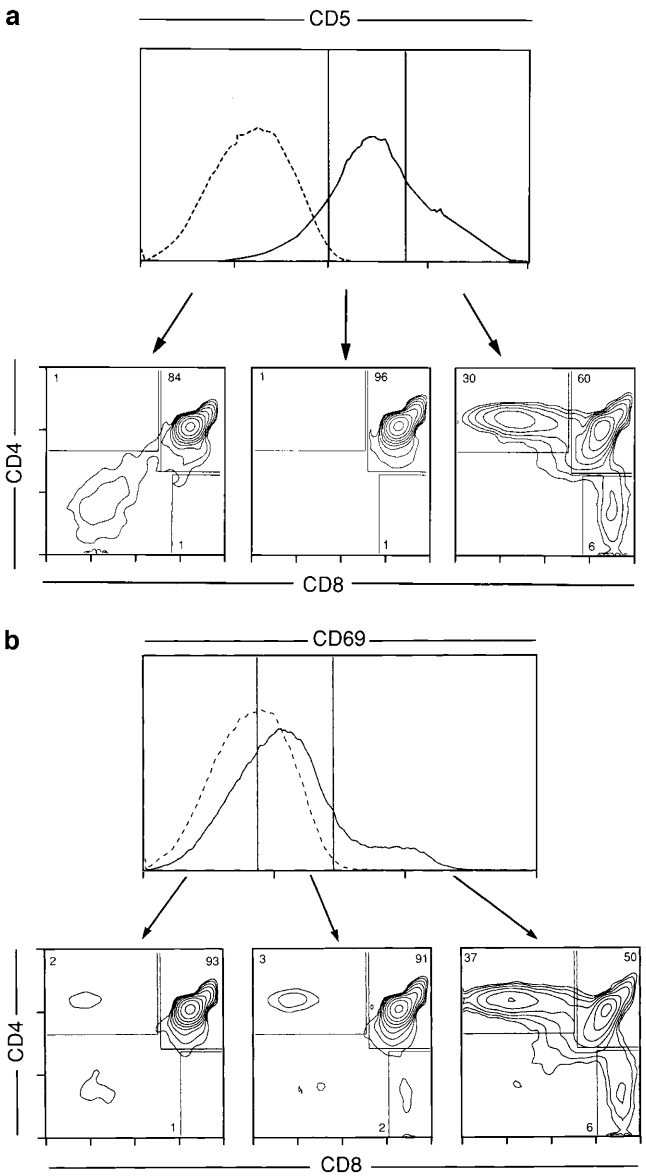

Figure 2. $CD4^+CD8^+$ thymocytes are heterogeneous for expression of CD5 and CD69. Freshly isolated thymocytes from B6 mice were analyzed by three color flow cytometry for surface expression of CD4, CD8 and CD5 (*a*) or CD69 (*b*). Gates subdividing cells into low, intermediate and high expression of CD5 (*a*) or CD69 (*b*) are indicated. Contour plots displaying CD4 and CD8 expression of cells in each gate are shown below each gate and the frequency of cells falling into $CD4^+CD8^+$, $CD4^-CD8^+$ and $CD4^+CD8^-$ subpopulations are indicated. Solid line indicates CD5 or CD69 staining and dashed line indicates negative control (Leu 4) staining.

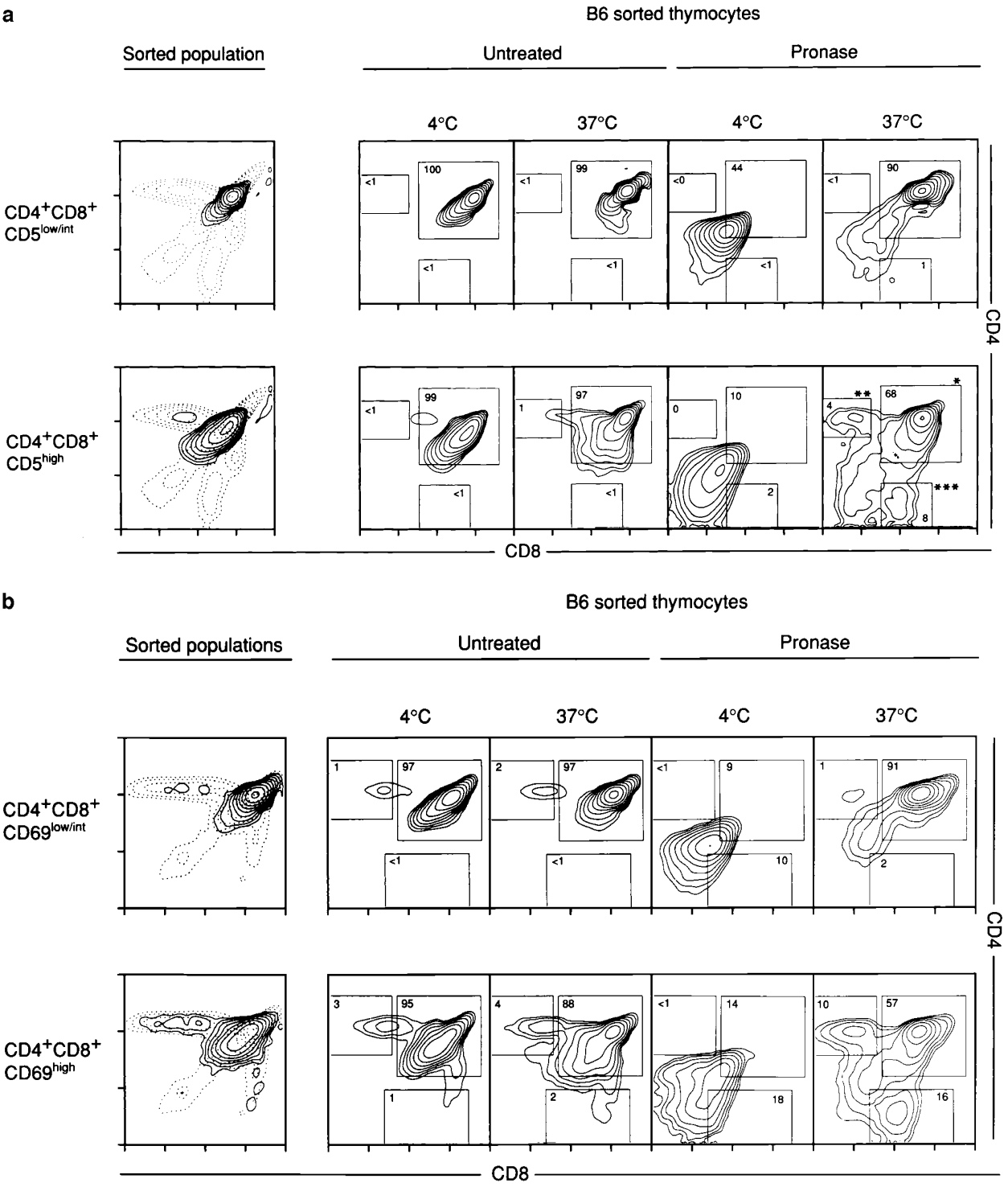

Figure 3. Coreceptor synthesis in $CD4^+CD8^+$ thymocytes. Populations of thymocytes from B6 mice were electronically sorted by three color flow cytometry into purified populations of $CD5^{hi}$ versus $CD5^{lo/int}$ $CD4^+CD8^+$ cells (*a*) or $CD69^{hi}$ versus $CD69^{lo/int}$ $CD4^+CD8^+$ cells (*b*). The sorting gates are indicated and the phenotype of the sorted cells after overnight culture at either 4°C or 37°C is shown (*left panels*). Sorted cells were assessed for coreceptor synthesis using the coreceptor reexpression assay, and the phenotype of pronase stripped cells after overnight culture at either 4°C or 37°C is shown (*right panels*). The frequency of cells falling into each boxed region is indicated. It is evident after pronase treatment and 37°C culture that $CD4^+CD8^+$ thymocytes that express only one coreceptor molecule are present only among the $CD5^{hi}$ (*a*) and $CD69^{hi}$ (*b*) $CD4^+CD8^+$ thymocyte subpopulations. The CD5 expression levels of sorted $CD5^{hi}$ $CD4^+CD8^+$ cells from (*a*) which only expressed CD8 (***), CD4 (**) or which continued to synthesize both coreceptors (*) were compared (*c*). Staining with a negative control antibody on unsorted populations is shown as a shaded histogram. This staining shows that the populations continuing to synthesize both CD4 and CD8 could not be distinguished from cells that had selectively terminated CD4 or CD8 synthesis by CD5 expression levels.

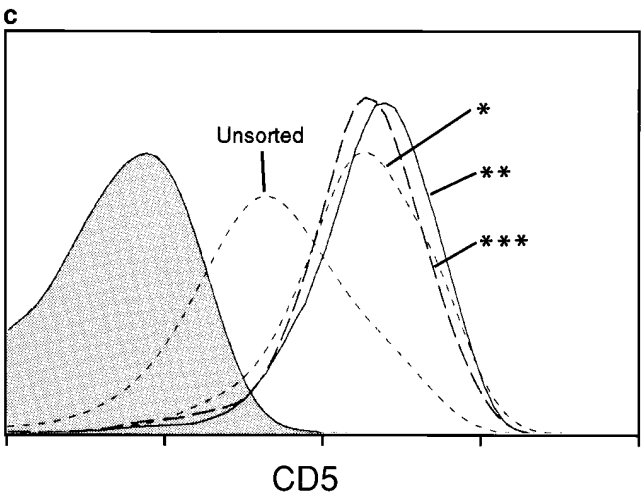

Figure 3. *Continued*

significant number of cells that have terminated or diminished synthesis of both CD4 and CD8. The fate of these cells is not yet known but they may represent precursors of mature DN T cells. Most importantly, these data indicate that cells selectively synthesizing only one coreceptor molecule (either CD4 or CD8) are not present among the vast majority of DP thymocytes, but are only present among DP thymocytes expressing high surface levels of CD5 and CD69.

Even though all DP thymocytes selectively synthesizing only one coreceptor molecule were $CD5^{hi}CD69^{hi}$, most of the cells that were $CD5^{hi}CD69^{hi}$ actively synthesized both coreceptor molecules (Fig. 3 *a*). Consequently, we wished to determine if markers existed that could uniquely identify $CD5^{hi}CD69^{hi}$ DP thymocytes that had selectively terminated synthesis of one coreceptor molecule and distinguish them from the majority of $CD5^{hi}CD69^{hi}$ DP thymocytes that continued to synthesize both coreceptors. As a first attempt we wished to quantitatively compare CD5 and CD69 surface expression on these different DP subpopulations. DP thymocytes that had undergone the coreceptor reexpression assay could be subsequently assessed for surface CD5 but not CD69 expression because CD5 is resistant to pronase treatment whereas CD69 is not. We found that surface CD5 expression was essentially equivalent on all $CD5^{hi}$ DP thymocytes, regardless of whether they were actively synthesizing one or both coreceptor molecules (Fig. 3 *c*) and therefore could not be used to distinguish the subpopulations.

However, we observed a clear difference when we examined the small subset of $CD5^{hi}CD69^{hi}$ DP thymocytes that had selectively terminated synthesis of one or the other coreceptor molecule for expression of two other differentiative markers, surface TCR and internal bcl-2 proteins (24–31) (Fig. 4). $CD5^{hi}$ DP thymocytes that were selectively synthesizing only one coreceptor molecule (either CD4 or CD8) expressed significantly higher levels of both surface TCRβ and internal bcl-2 protein than $CD5^{hi}$ DP thymocytes that continued to synthesize both coreceptor molecules (Fig. 4). Thus, DP thymocytes that have selectively terminated synthesis of one coreceptor molecule (either CD4 or CD8) are exclusively contained within a small subpopulation of DP thymocytes that are not only $CD5^{hi}CD69^{hi}$, but are also $TCR^{hi}bcl\text{-}2^{hi}$.

These results demonstrate that selective termination of synthesis of one coreceptor molecule is a stringently regulated event during development which is evident only within the small, most differentiated subset of DP thymocytes defined as $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$. In addition, these results identify three subsets of DP thymocytes that differ in their state of coreceptor synthesis: (*a*) $CD5^{lo}$ $CD69^{lo}$ DP thymocytes that actively synthesize both coreceptor molecules; (*b*) $CD5^{hi}CD69^{hi}TCR^{lo}bcl2^{lo}$ DP thymocytes that synthesize both coreceptor molecules; and (*c*) $CD5^{hi}CD69^{hi}TCR^{hi}bcl2^{hi}$ DP thymocytes that actively synthesize only one coreceptor molecule having terminated expression of the other (Table 1). We propose there may be a precursor/progeny relationship among these subpopulations such that they define the progression of DP thymocytes from uncommitted to precommitted to lineage-committed thymocytes.

Selective Termination of Synthesis of One Coreceptor Molecule Requires Surface TCRαβ/CD3 Complexes and Occurs Late in Development. As an independent test of our conclusion that selective termination of coreceptor synthesis is a developmentally regulated event that only occurs in the most differentiated subset of $CD5^{hi}$ DP thymocytes, we exam-

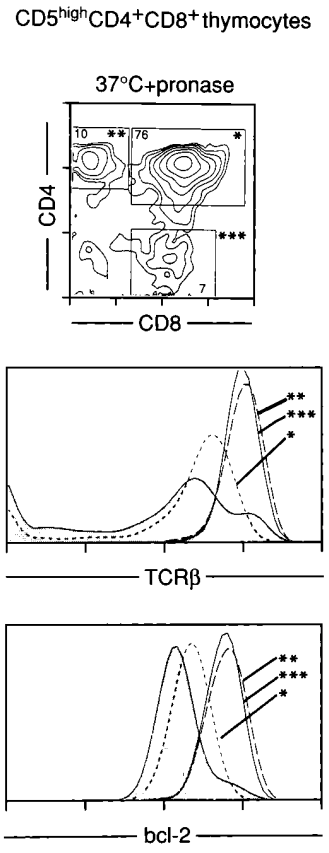

Figure 4. TCRβ and bcl-2 expression identify $CD4^+CD8^+$ thymocytes that have selectively terminated synthesis of one coreceptor molecule. Purified $CD5^{hi}$ $CD4^+CD8^+$ thymocytes were obtained by electronic sorting and assessed by the coreceptor reexpression assay. The CD4 and CD8 profile of sorted $CD5^{hi}$ $CD4^+CD8^+$ cells treated with pronase and cultured overnight at 37°C is shown as a contour plot. TCRβ and bcl-2 expression of sorted cells which only reexpressed CD8 (***), CD4 (**) or which continued to synthesize both CD4 and CD8 (*) are displayed. The shaded profiles represent staining of unsorted whole thymocytes that had been similarly pronase treated and cultured at 37°C overnight.

ined DP thymocyte populations from TCR$\alpha^{-/-}$ mice that are unable to assemble and express surface TCR$\alpha\beta$ complexes and, consequently, lack CD5^{hi} DP thymocytes (Fig. 5 *a*). In this experiment, we attempted to increase our ability to detect DP thymocytes synthesizing only one coreceptor molecule by performing the coreceptor reexpression assay on purified populations of CD4^{+}8^{lo} and CD4^{lo}8^{+} transitional DP thymocytes that may be developmentally more advanced than the bulk of DP thymocytes (Fig. 5 *b*). Even so, we failed to detect any DP thymocytes in TCR$\alpha^{-/-}$ mice that were selectively synthesizing only one coreceptor molecule (Fig. 5 *b*). Thus, surface expression of TCR$\alpha\beta$/CD3 complexes and the signals they transduce are required for generation of CD5^{hi} DP thymocytes and for selective termination of a coreceptor molecule.

Having confirmed that selective termination of coreceptor synthesis occurs only in CD5^{hi} DP thymocytes, we wished to examine the developmental relationship between CD5^{hi} DP thymocytes that continue to synthesize both coreceptor molecules and those that have selectively terminated coreceptor synthesis. In particular, if CD5^{hi} DP thymocytes that continue to synthesize both coreceptor molecules are the precursors of those that have selectively terminated synthesis of one coreceptor molecule, it would be expected that these populations would appear sequentially in development. To examine this possibility, we performed the coreceptor reexpression assay on CD5^{hi} DP thymocytes from newborn mice less than 12 h old (Fig. 6). Interestingly, newborn mice contained CD5^{hi} DP thymocytes at a frequency comparable to that of adult mice (10–15% of all DP cells). However, essentially all neonatal CD5^{hi} DP thymocytes actively synthesized both coreceptor molecules; very few, if any, had terminated synthesis of either or both coreceptor molecules (Fig. 6). Thus, CD5^{hi} DP thymocytes that synthesize both coreceptor molecules appear earlier in development than CD5^{hi} DP thymocytes that synthesize only one coreceptor molecule, consistent with (but not proving) a precursor/progeny relationship.

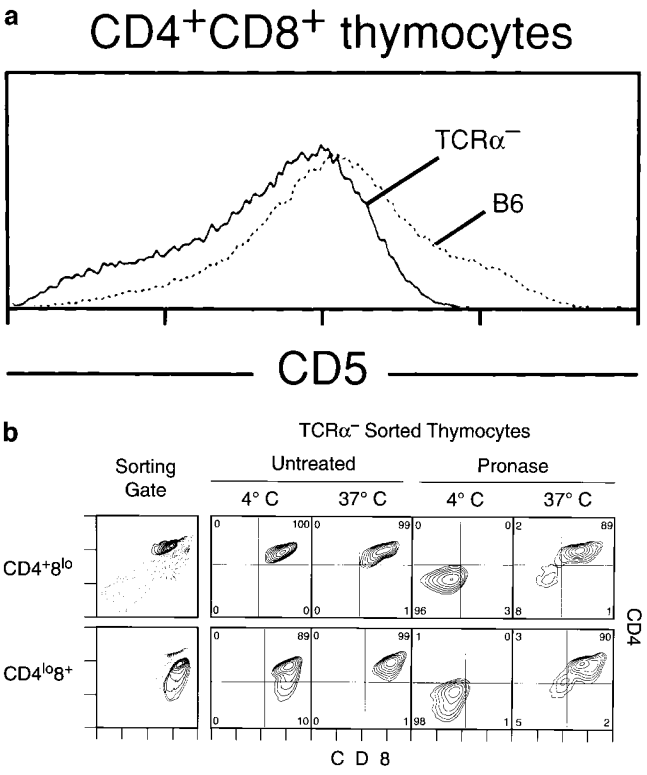

Figure 5. Selective termination of coreceptor synthesis requires TCR$\alpha\beta$/CD3 expression. (*A*) CD5 expression of CD4^{+}CD8^{+} thymocytes from wild-type and TCRα deficient mice was assessed by flow cytometry. It is evident that the CD5^{hi} CD4^{+}CD8^{+} thymocyte subpopulation is absent in TCRα-deficient mice. (*B*) CD4^{+}CD8^{lo} and CD4^{lo} CD8^{+} subpopulations of thymocytes from a TCRα-deficient mouse were electronically sorted according to the gates indicated and were assessed for coreceptor synthesis by the coreceptor reexpression assay. Frequency of cells falling into each quadrant are indicated. No cells that had selectively terminated CD4 or CD8 synthesis are evident in either population.

Discussion

Lineage commitment is a developmental process by which positively selected DP thymocytes are induced to differentiate into either CD4^{+} or CD8^{+} T cells. Identification of the intrathymic signals that induce lineage commitment would be significantly facilitated if the molecular consequences of lineage commitment could be recognized as soon as they occurred in DP thymocytes. We have proposed that lineage commitment be molecularly defined as the selective termination of synthesis of one or the other coreceptor molecule in a DP thymocyte expressing both coreceptors on its surface. Lineage commitment by any definition must require that DP thymocytes shift from synthesizing both coreceptor molecules to synthesizing only

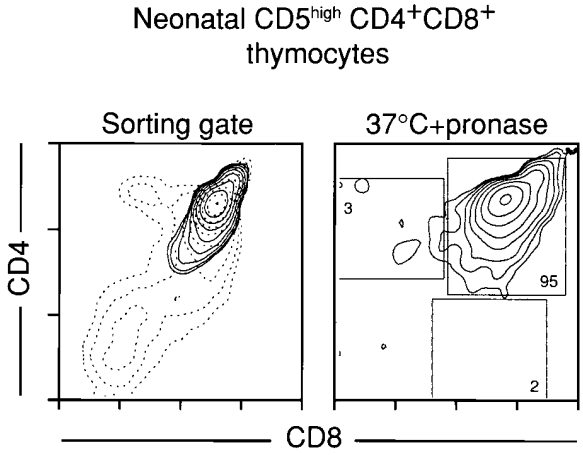

Figure 6. CD5^{hi} CD4^{+}CD8^{+} cells within a newborn thymus have not yet selectively terminated CD4 or CD8 synthesis. Neonatal thymi were harvested within 12 h of birth and CD5^{hi} CD4^{+}CD8^{+} cells were electronically sorted according to the gate indicated in the left panel. The coreceptor reexpression assay was performed on the sorted subpopulation and the frequency of cells falling into each quadrant is indicated. It is evident that the vast majority of CD5^{hi} CD4^{+}CD8^{+} thymocytes continued to synthesize both CD4 and CD8; no discrete populations of cells that had selectively terminated CD8 or CD4 synthesis was evident. (For comparison, Fig. 4 displays an experiment performed with CD5^{hi} CD4^{+}CD8^{+} thymocytes from adult mice.)

one. However, the validity of defining lineage commitment in DP thymocytes by coreceptor synthesis has been questioned because of the possibility that selective termination of coreceptor synthesis may occur randomly in DP thymocytes and, therefore, may be unrelated to their differentiation state.

The present study, however, demonstrates that selective termination of CD4 or CD8 synthesis as determined by the coreceptor reexpression assay does not occur randomly in DP thymocytes, but, instead is a highly regulated event which has occurred exclusively within a developmentally discrete subpopulation of DP thymocytes that is $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$. DP thymocytes expressing high levels of CD69, TCR and bcl-2 have each been shown by other investigators to have undergone positive selection (19–20, 24–30) and TCR^{hi} subpopulations of DP thymocytes, specifically, have been shown to contain the immediate precursors of SP thymocytes (23–26). That termination of coreceptor synthesis was only evident within that population of thymocytes which has undergone positive selection is compelling evidence that selective termination of coreceptor synthesis is not a capricious event in DP thymocytes but is a molecular indicator of lineage commitment. Importantly, selective termination of coreceptor synthesis as detected by the coreceptor reexpression assay, unlike other markers identifying lineage committed DP thymocytes, reveals the T cell lineage to which individual DP thymocytes are committed since it reveals the coreceptor molecule that committed thymocytes continue to synthesize.

The present study has identified three distinct subpopulations of DP thymocytes that are distinguishable by surface phenotype and lineage commitment status: (*a*) $CD5^{lo}$ $CD69^{lo}$ DP thymocytes that have not selectively terminated synthesis of either CD4 or CD8 coreceptor molecules, (*b*) $CD5^{hi}CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes that continue to synthesize both coreceptor molecules, and (*c*) $CD5^{hi}CD69^{hi}$-$TCR^{hi}bcl\text{-}2^{hi}$ DP thymocytes that actively synthesize only one or the other coreceptor molecule, but not both. A striking and unanticipated finding of the present study is that all DP thymocytes that have selectively terminated either CD4 or CD8 synthesis are $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$ and, furthermore, that essentially all $CD5^{hi}CD69^{hi}TCR^{hi}$-$bcl\text{-}2^{hi}$ DP thymocytes have selectively terminated either CD4 or CD8. This precise overlap suggests that intrathymic signals induce the immediate precursors of these lineage committed DP thymocytes (presumed to be $CD5^{hi}CD69^{hi}$-$TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes) to coordinately: (*a*) upregulate TCR expression, (*b*) increase bcl-2 content, and (*c*) selectively terminate synthesis of one coreceptor molecule. Consequently, $CD5^{hi}CD69^{hi}TCR^{int}bcl\text{-}2^{lo}$ DP thymocytes synthesizing both coreceptor molecules appear to be the targets of lineage commitment signals in the thymus. A summary of these findings is presented in Table 1.

We think that a precursor/progeny relationship exists among all three DP thymocyte subpopulations which is consistent with their sequential appearance during development, although it is difficult to prove such precursor/progeny relationships conclusively. From a lineage commitment perspective, we consider $CD5^{lo}CD69^{lo}$ DP thymocytes to be uncommitted; $CD5^{hi}CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes to be potential targets of lineage commitment signals and so are precommitted; and $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$ DP thymocytes to be committed. The differentiation of $CD5^{lo}$ $CD69^{lo}$ DP thymocytes into $CD5^{hi}CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes presumably requires intrathymic signals transduced by surface TCR/CD3 complexes as CD5 and CD69 upregulation is mediated by TCR/CD3 signaling (17, 20, 32). Notably, the present study indicates that such intrathymic TCR/CD3 signals are not sufficient to selectively terminate synthesis of either CD4 or CD8 coreceptor molecules, as $CD5^{hi}CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes continue to synthesize both coreceptor molecules. Rather, we think additional signals are required in $CD5^{hi}$ $CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes to increase TCR expression, to increase bcl-2 expression, and to selectively terminate either CD4 or CD8 coreceptor synthesis. It is important to note, however, that the present data indicate that TCR/CD3 signals are necessary for selective termination of CD4 or CD8 synthesis. Whether these signals are generated by TCR engagement by antigen or by ligand engagement of non-clonotypic receptors that signal via the TCR/CD3 signaling complex, such as Thy-1 (33), is not known.

In the present study the coreceptor reexpression assay has been performed on DP thymocytes, whereas we have previously utilized the coreceptor assay on transitional populations of DP thymocytes that have quantitatively downregulated surface expression of one or the other coreceptor molecule (12). The results of our previous studies indicated

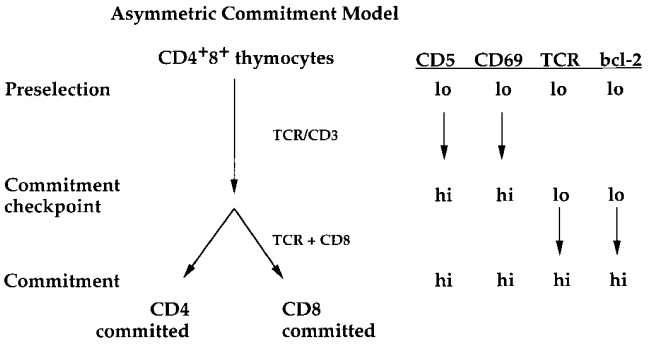

Figure 7. Subpopulations of $CD4^+CD8^+$ thymocytes, their commitment status, and their relationship to the Asymmetric Commitment Model (12). The results of the present study provide a phenotype that can be assigned to each of the commitment stages proposed in the asymmetric commitment model (12). Preselection $CD4^+CD8^+$ thymocytes are too immature to undergo lineage commitment and express low levels of CD5, CD69, TCR and bcl-2. Signals transduced by the TCR/CD3 complex (as result of engagement of those surface molecules that signal through the TCR/CD3 complex, e.g. TCR, Thy-1, etc.) increase CD5 and CD69 expression and promote the differentiation of preselection $CD4^+CD8^+$ thymocytes to the point that they are able to undergo lineage commitment (commitment checkpoint). At the commitment checkpoint TCR-signaled $CD4^+CD8^+$ thymocytes whose TCR and CD8 molecules are coengaged become CD8-committed, whereas TCR-signaled $CD4^+CD8^+$ thymocytes whose TCR and CD8 molecules are not coengaged become CD4-committed. Upregulation of TCR and bcl-2 occurs coincident with lineage commitment so that lineage committed thymocytes express TCR and bcl-2 at high levels.

that termination of CD4 synthesis was more stringently regulated during development than termination of CD8 synthesis, and we advanced a model, the asymmetric commitment model, to explain our findings. Specifically, we proposed that DP thymocytes progress to a stage in development (termed the commitment checkpoint) when they are assessed for the presence or absence of a CD8-commitment signal. The presence of a CD8-commitment signal results in selective termination of CD4 synthesis (CD8-commitment), whereas the absence of a CD8-commitment signal results by default in selective termination of CD8 synthesis (CD4-commitment). The present study provides a phenotype that can be assigned to each of the commitment stages in the asymmetric commitment model (Fig. 7): $CD5^{lo}CD69^{lo}$ DP thymocytes are uncommitted cells that are preselection; $CD5^{hi}CD69^{hi}TCR^{lo}bcl\text{-}2^{lo}$ DP thymocytes are cells at the commitment checkpoint and so are precommitment; $CD5^{hi}CD69^{hi}TCR^{hi}bcl\text{-}2^{hi}$ DP thymocytes have committed to either the CD4 or CD8 T cell lineages (Fig. 7). However, the present finding that all lineage-committed DP thymocytes, even those that have selectively terminated CD8 synthesis, are $CD5^{hi}CD69^{hi}$ indicates that commitment to either lineage occurs only in TCR/CD3 signaled DP thymocytes. Hence, we would like to clarify the asymmetric commitment model to state that: (*a*) DP thymocytes that fail to receive TCR/CD3 signals remain $CD5^{lo}CD69^{lo}$ and die from neglect as uncommitted DP thymocytes; and (*b*) CD4-commitment occurs in $CD5^{hi}CD69^{hi}$ DP thymocytes that have not received CD8-commitment signals.

In conclusion, the present study demonstrates that selective termination of CD4 or CD8 coreceptor synthesis in DP thymocytes is a highly regulated developmental process that occurs in strict parallel with other events that define positive selection. It is anticipated that the molecular definition of lineage commitment validated in this study will enhance identification of the intrathymic signals that induce lineage commitment in developing thymocytes.

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