**Summary**

The tyrosine kinase p56lck regulates the differentiation and proliferative expansion of pre-T cells. However, nothing is known about other signaling molecules that operate with p56lck to mediate the pleiotropic changes that occur at this stage of thymocyte development. We used a genetic strategy to examine the requirement for the GTPase Rho in p56lck-mediated signals in the thymus. By generating mice double transgenic for a constitutively activated form of p56lck (p56lck\textsuperscript{F505}) and the Rho inhibitor C3 transferase we were able to compare thymocyte development in mice expressing active p56lck on a wild-type or Rho\textsuperscript{2} background. Thymocytes expressing active p56lck show enhanced proliferation of pre-T cells resulting in increased numbers of late pre-T cells, however, this dramatic effect on pre-T cell proliferation is lost when the p56lck transgene is expressed in thymocytes lacking endogenous Rho GTPase function. Expression of active p56lck also generates double positive (DP) thymocytes with low levels of CD2 antigen expression. Again, p56lck cannot prevent expression of CD2 when expressed on a Rho\textsuperscript{2} background. CD4\textsuperscript{+}CD8\textsuperscript{+} DP cells expressing active p56lck have been shown to lack functional \(\alpha/\beta\)-T cell receptor (TCR) complexes due to p56lck-mediated inhibition of TCR gene \(V_{\beta}-D_{\beta}\) rearrangement. This inhibition of TCR expression by active p56lck is unimpaired in the absence of Rho function. The signaling pathways that are mediated by p56lck and control thymocyte proliferation, \(\alpha/\beta\)-TCR and CD2 antigen expression can thus be distinguished by their dependency on Rho function.

Key words: pre-T cell • p56lck • Rho • signaling • development
protein tyrosine kinases. Mouse strains with null mutations in the tyrosine kinases p56lck (6), ZAP70, and Syk (7), or their regulatory molecules such as CD45 (8) show defects at the pre-T cell stage of development. Several other studies in particular highlight the importance of the tyrosine kinase p56lck in the transmission of pre-TCR-mediated signals. Mice lacking p56lck or overexpressing a dominant inhibitory p56lck mutant (6, 9) show a defect in early thymopoiisis similar to that seen in pTα or TCR-β chain-deficient mice. Additionally, transgenic expression of a constitutively active form of p56lck can induce the transition of thymocyte progenitors from the CD44−CD25− to the CD44+CD25+ compartment and induce the generation of DP thymocytes in mice lacking expression of the pre-TCR complex, e.g., in RAG−/− or pTα−/− mice (10, 11). This indicates that activated p56lck is sufficient to initiate all the critical events that occur as CD44−CD25− early pre-T cells develop into CD44+CD25− late pre-T cells: the onset of rapid cell cycle progression, allelic exclusion at the TCR-β chain locus and ultimately expression of CD4 and CD8.

We recently generated mice that lack function of the GTPase Rho in thymocytes due to tissue-specific transgenic expression of a Rho inhibitor, C3 transferase, which selectively ADP-ribosylates Rho and thereby abolishes its biological activity (12). The phenotype of these mice indicated that Rho might also be important for pre-TCR function (13). Mice lacking Rho function in the thymus were able to generate DP and SP thymocytes, but at severely reduced levels. Loss of Rho function resulted in decreased cell proliferation in CD44−CD25− late pre-T cells due to a partial block in G1/S cell cycle progression. This stage of thymocyte development is regulated by p56lck which raises questions about the positioning of Rho in the context of the p56lck signals that control the proliferation of early thymocyte progenitors. In fact, very little is known about the signaling pathways downstream of p56lck that control pre-T cell differentiation. Given the involvement of both p56lck and the GTPase Rho in the regulation of proliferation in late pre-T cells we considered it possible that Rho might function in the signaling pathways used by p56lck to regulate the checkpoints for proliferation and differentiation at the pre-T cell stage of thymocyte development. To explore these questions, we generated mice double transgenic for active p56lck (p56lckF505, reference 14) and C3 transferase (12) and examined the ability of active p56lck to drive thymocyte proliferation and differentiation in the absence of Rho function. To explore these questions, we generated mice double transgenic for active p56lck (p56lckF505, reference 14) and C3 transferase (12) and examined the ability of active p56lck to drive thymocyte proliferation and differentiation in the absence of Rho function. Mice expressing active p56lck show accelerated pre-T cell proliferation and generate CD4−CD8− DP thymocytes that fail to express α/β-TCR complexes via a process that mimics allelic exclusion. The present study reveals that the proliferative signals generated by p56lck are suppressed in mice lacking thymic Rho function. In contrast, the ability of active p56lck to suppress expression of α/β-TCR complexes is unimpaired. p56lck thus uses either qualitatively or quantitatively different signals to control thymocyte development at the pre-T cell stage, p56lck-mediated signaling pathways for proliferation are dependent on the Rho GTPase whereas signals for regulation of antigen receptor expression are not.

Materials and Methods

Transgenic Mice. Transgenic mice expressing the C3 trans- ferase gene under the control of the proximal p56lck promoter were generated as previously described (12). Mice transgenic for an activated form of the tyrosine kinase p56lck (p56lckF505; strain pLGF-296A, 10 copies of p56lckF505 transgene) were provided by Dr. Roger M. Perlmutte (Merck Research Labs.; 14). Expression of this transgene is also driven by the proximal p56lck promoter, restricting expression of the transgene to thymocytes. Mice were kept under SPF conditions and bred to C57BL/6/j mice to maintain the transgenic lines. Mice double transgenic for C3 transerase and p56lckF505 (pLGF/C3) were generated by cross-breeding of the parental strains.

PCR. Offspring were genotyped for the presence of both transgenes by PCR analysis of genomic mouse DNA using trans- gene specific primer pairs: b-actin (actin 1: 5′-GTGGC CATCCTCGTCTGAAGTC-3′ and actin 2: 5′-GTGGTACACCTTCA-ACACCCC-3′); C3 transerase (G9112: 5′-GCCCACATGAGCAGGAGAACG-TCATCCCG-3′ and 3CNT: 5′-CTGATT-TGCTTTAGTCCTAC-3′); and p56lckF505 (pLGF-Fwd: 5′-ATGACTTTCTTCAACGCCAGAAGG-3′ and pLGF-Rev: 5′-TTTTTATTAGGCAAGCGGCTG-GGC-3′).

PCR reactions were performed in 20 mM ammonium sul- fate, 75 mM Tris-HCl, pH 9.0, 0.01% Tween 20, 1.2 mM MgCl2, 200 μM dNTP, 0.5 U Taq DNA polymerase, and 1 μM of each primer. Genomic DNA was purified from mouse ear clips and used as template for 30 cycles of polymerase chain reaction (57°C annealing, 72°C elongation, 95°C denaturation). PCR products were separated in 2% agarose gels and stained with ethidium bromide.

Thymocyte Preparation. Young adult mice of ~4–6 wk of age were typically analyzed. Thymocytes were obtained by carefully mincing the thymus with forceps and filtering through a fine mesh filter to obtain a single cell suspension. Total cell numbers were determined by counting of trypan blue stained thymocytes within a representative volume of cell suspension using a Nue- bauer hemocytometer.

Flow Cytometric Analysis. Freshly isolated thymocytes were stained with saturating concentrations of antibody in 100 μl cold PBS supplemented with 1% BSA on ice for 30 min in a 96-well V-bottom shaped microtiter plate. Cells were washed twice in between incubations with this buffer. In a first step cells were incubated with anti-FcgRII mAb in order to block any unspecific staining. All mAb used were conjugated to either FITC, PE, or biotin. Biotinylated mAb were revealed using streptavidin-Tricolor (Caltag Laboratories, South San Francisco, CA). The fol- lowing mAb were used (all PharMingen, San Diego, CA): CD8 (53-5-8), CD4 (R-4-5), CD25 (IL2R α chain, 3C7), CD3ε (145-2C11), α/β-TCR (β chain, H57-597), B220 (CD45R, R-6-3-B2), CD44 (Pgp-1, IM7), CD2 (LFA-2, R-2-5-M), Mac-1 (alk chain, M1/70), Gr-1 (Ly-6G, RB6-8C5), and NK (2B4, 129/J). Cells stained negative for CD4, CD8, CD25, Gr-1, Mac-1, and the NK-cell marker 2B4 were labeled Lin−. Stained thymocytes were analyzed on a FACScan® Calibur (Becton Dickinson & Co., Sparks, MD) using CellQuest software (Becton Dickens- on & Co.). Viable cells were gated on the basis of forward and side light scatter.

Cell Cycle Analysis. Cellular DNA content was assayed by standard techniques using staining with propidium iodide. In brief, thymocytes were stained with biotinylated mAb against CD4, CD8, CD3, B220, NK, Mac-1, Gr-1, and CD44 and surface staining revealed with FITC conjugated Avidin (Sigma Chemical Co., St. Louis, MO). Cells were fixed in 70% ethanol
for 30 min on ice, washed free of ethanol, RNase (1 mg/ml) treated for 15 min at room temperature, and then resuspended in propidium iodide (50 ug/ml in PBS) in order to stain DNA. Maintaining a low flow rate events were collected and propidium iodide fluorescence was measured >600 nm on a FACS Calibur (Becton Dickinson & Co.). DNA content of FITC-negative (CD44 -Lin-) thymocytes was analyzed using a doublet discrimination module.

Results

Loss of Endogenous Rho Function Inhibits p56lck-mediated Proliferative Responses and Developmental Maturation in Pre-T Cells. The developmental maturation of pre-T cells is associated with downregulation of the CD25 surface marker and the acquisition of CD4 and CD8 molecules (15). At this stage, only thymocytes that productively rearranged their TCR-β chain and express a complete pre-TCR complex are selected for further development and transition into the DP compartment, a process known as β-selection (16, 17). Current models propose that the pre-TCR complex triggers this maturation program via intracellular signaling pathways that involve activation of the tyrosine kinase p56lck (18). β-selection is bypassed in thymocytes that express a constitutively activated form of p56lck (19), resulting in differentiation and proliferation of all CD44+CD25+ pre-T cells, regardless of a productive or unproductive β chain rearrangement. Accordingly, pLGF mice expressing a constitutively active p56lck (p56lckT520S) transgene in the thymus show enhanced generation of CD44+CD25+ pre-T cells compared with normal control mice (10). Comparison of CD25 expression on CD44+ triple negative (TN) thymocytes (CD44+CD4−CD8−CD3−) from pLGF mice with normal control mice shows how expression of activated p56lck results in an increased frequency of CD44+CD25− late pre-T cells and a concomitant low frequency of CD44+CD25+ early pre-T cells (Fig. 1 a).

Analysis of the cellular DNA content of pre-T cells from pLGF mice compared with normal control mice is shown in Fig. 1 b. We observed a marked increase of cells in the proliferative S and G2 phases of the cell cycle in CD44−TN thymocytes from pLGF mice when compared with cells of the same phenotype from normal control mice. Thymocyte-specific transgenic expression of activated p56lck thus accelerates proliferative responses in pre-T cells. The dramatic enhancement of the production of late pre-T cells by active p56lck is illustrated by a comparison of total cell numbers for this population in pLGF mice compared with control mice. A normal thymus contains an average number of CD44+CD25− late pre-T cells of 1.2 × 106, whereas in pLGF mice their numbers are typically increased to ~6.0 × 106. There is thus a fivefold increase in total numbers of CD44+CD25− late pre-T cells in thymi from pLGF mice compared with thymi from normal control mice.

Proliferative responses in late pre-T cells are reduced in thymi lacking endogenous Rho function as a consequence of transgenic expression of C3 transerase (C3). These cells show a severe decrease in the production of late pre-T cells (12). To explore whether p56lck-mediated responses in early T cell development required endogenous Rho function we crossed pLGF mice (14) to transgenic mice with inactivated thymic Rho function (C3). We then used CD44 and CD25 markers to analyze early thymocyte progenitors in TN thymocytes of pLGF/C3 double transgenic mice. Total cell numbers and the cell cycle status of the CD44+CD25− pre-T cell compartment were monitored. The data in Fig. 2 a show that the increases in the percentage of cycling pre-T cells seen in pLGF mice are lost in pLGF/C3 double transgenic mice, i.e., loss of Rho function antagonizes the action of p56lck in driving cell cycle progression in pre-T cells. There are still some cycling cells in pre-T cells from pLGF/C3 double transgenic mice but the loss of Rho

![Figure 1. Transgenic expression of activated p56lck enhances differentiation and proliferation of pre-T cells. (A) CD25 expression profiles of CD44+TN thymocytes in normal control mice and pLGF transgenic mice expressing active p56lck. Single cell suspensions of total thymocytes from normal control and pLGF transgenic mice were prepared and stained with mAb against a panel of mature lineage markers including CD4, CD8, CD3, B220, NK, Gr-1, M-ac-1 (all biotinylated), CD44 (biotinylated), and CD25 (FITC). Cells negative for all lineage markers and CD25 were gated and examined for their CD25 expression profile. (B) Transgenic expression of active p56lck leads to increased levels of CD44+TN thymocytes in S+G2/M phase of the cell cycle. Total thymocytes from normal and pLGF transgenic mice were prepared and stained with mAb against CD44 (biotinylated) and a panel of mature lineage markers including CD4, CD8, CD3, B220, NK, Gr-1, and Mac-1 (all biotinylated), fixed in 70% ethanol, and stained with propidium iodide (PI). Cells lacking expression of CD44 and all lineage markers were gated and then examined for their DNA profile.](image-url)
function appears to prevent the ability of activated p56lck to induce a hyperproliferative response in CD44−CD25− late pre-T cells.

The most striking consequence of the decreased proliferative activity of pre-T cells in pLGF/C3 double transgenic mice is presented in Fig. 2b, which shows absolute numbers of CD44−CD25− late pre-T cells in pLGF and pLGF/C3 mice. The data reveal that in the absence of endogenous Rho function p56lck is unable to enhance the production of CD44−CD25− late pre-T cells. Thus, pLGF/C3 double transgenic mice do not show the skewed distribution of CD44−CD25− and CD44−CD25+ subpopulations seen in pLGF mice. These results indicate that Rho function is required for active p56lck to accelerate the transition of early thymocytes from the CD44−CD25+ to the CD44−CD25− compartment.

p56lck-mediated Inhibition of α/β-TCR Expression and Generation of CD4+ or CD8+ SP T cells is Not Rho Dependent. The consequence of a productive rearrangement of the TCR-β locus in thymocytes is expression of the pre-TCR complex and the initiation of the process of allelic exclusion that ensures that mature T cells express a TCR complex with only one β chain (20). p56lck function is required for this pre-TCR initiated response. Moreover, transgenic expression of activated p56lck inhibits V-DJ β-rearrangements and expression of the TCR-β chain in thymocytes. Consequently, DP thymocytes that develop in pLGF mice show defective expression of the TCR-CD3 complex with only one β chain (19). The data in the left panel of Fig. 3a show α/β-TCR staining profiles of thymocytes from pLGF mice in comparison with thymocytes from control littermate mice. Normal thymocytes contain a large subpopulation of cells expressing intermediate levels of the TCR-CD3 complex and a smaller subset of cells with upregulated, higher levels of TCR expression. In contrast, thymocytes derived from pLGF mice show an abnormal α/β-TCR staining pattern with few intermediate or high α/β-TCR positive cells.

If Rho function was required for p56lck-mediated suppression of TCR-β chain rearrangements then expression of activated p56lck in Rho− thymocytes would not prevent expression of the α/β-TCR complex. Thymocytes from pLGF/C3 double transgenic mice should then express a mature α/β-TCR complex, undergo positive selection, and generate mature SP T cells in the normal ratio. Conversely, if Rho function was not required for p56lck-mediated regulation of α/β-TCR expression then pLGF/C3 thymocytes should show a pattern of TCR expression and CD4/CD8 differentiation like that seen in pLGF thymocytes that express active p56lck alone. Therefore, we analyzed expression of the TCR-CD3 complex and the development of CD4 and CD8 mature SP cells in thymocytes from pLGF/C3 double transgenic mice. As shown in the right panel of Fig. 3a, pLGF/C3 thymocytes that express active p56lck on a Rho− background fail to normally express a mature TCR-CD3 antigen receptor complex.

The absence of a mature TCR-CD3 complex interrupts the normal processes of positive and negative selection and inhibits CD4 and CD8 SP development in the thymus of pLGF mice. As shown in the upper right panel of Fig. 3b, thymocytes expressing active p56lck comprise CD4−CD8− DN and CD4+CD8− DP cells but abnormally low levels of CD4+ or CD8+ SP cells. We have shown previously that positive and negative selection processes of DP thymocytes occur undisturbed in the absence of Rho function (12). Adult thymy which lack endogenous Rho function show severe hypopcellularity of only ~5–10% of normal cell numbers but do contain DP and mature SP thymocytes with normal expression levels of the TCR-CD3 complex (Fig. 3b, lower left). The lower right panel in Fig. 3b shows the result of a representative analysis of CD4/CD8 subpopulations in pLGF/C3 thymocytes. The frequencies of DP and mature SP cells of thymocytes from pLGF/C3 double transgenic mice are identical to that of thymocytes from pLGF mice. The data in Fig. 3 thus show that introduction of an activated p56lck transgene into thymocytes lacking endogenous Rho function prevents the expression of the α/β-TCR complex and consequently, because positive se-
lection can not occur, the generation of mature CD4 and CD8 SP thymocytes. In this context, the outcome of expression of activated p56

\( \text{p} \)\text{56lck in terms of inhibition of } \alpha/\beta-\text{TCR complexes and suppression of the differentiation of CD4 and CD8 SP cells is the same in Rho-}^\text{2} \text{ and normal thymocytes.}

Immature CD8 single positive cells (ISP), distinguished by low levels of CD8 antigen expression but no expression of the TCR–CD3 complex, represent an intermediate stage in the transition from the CD4–CD8– DN to the CD4+CD8+ DP compartment (21, 22). These cells are not easy to discern in normal thymocytes but can accumulate in mice where there is a partial block or a delay in the DN to DP transition. For example, an accumulation of CD8\text{low}CD3– ISPs was seen in mice lacking expression of the transcription factors Tcf-1 and Lef-1 (23). A consistent and reproducible observation was that thymocytes from pLGF/C3 double transgenic mice contained higher frequencies of CD8\text{low} ISPs when compared with pLGF single transgenic mice (Fig. 3c).

Activated p56lck Regulates Expression of CD2 by a Rho-dependent Pathway. In normal thymocyte development pre-TCR–mediated downregulation of CD25 expression is accompanied by upregulation of the expression of the CD2 antigen (24, 25). The data in Fig. 4a show CD2 expression of thymocytes isolated from a normal young C57/BL6 mouse. CD2 expression is found on CD4+CD8+DP and SP thymocyte populations. There is a subtle but clear regulation of CD2 expression levels during normal thymocyte development in that SP thymocytes express about threefold higher levels of the CD2 antigen than DP thymocytes.

Figure 3. Failed expression of \( \alpha/\beta-\text{TCR complexes and development of mature SP cells in thymocytes expressing active p56lck is independent of endogenous Rho function. (A) } \alpha/\beta-\text{TCR expression in normal and Rho-}^\text{2} \text{ thymocytes expressing active p56lck. Total thymocytes from normal control mice, pLGF transgenic and pLGF/C3 double transgenic mice were stained for } \alpha/\beta-\text{TCR expression (anti–TCR- } \beta\text{-chain-FITC) and TCR expression profiles of transgenic mice (shaded) were compared with normal profiles (outline) by overlay. (B) Expression of CD4 and CD8 markers in normal and Rho-}^\text{2} \text{ thymocytes expressing active p56lck. Representative dual parameter histograms for CD4 and CD8 expression of total thymocytes isolated from adult normal, C3 and pLGF transgenic and pLGF/C3 double transgenic mice. Percentages of individual CD4/CD8 subpopulations are indicated. Data shown are representative of four such experiments. (C) Frequencies of CD4+CD8\text{low} cells in normal and Rho-}^\text{2} \text{ thymocytes expressing active p56lck. Three representative dual parameter histograms for CD4 and CD8 expression of total thymocytes isolated from adult pLGF transgenic and pLGF/C3 double transgenic mice are shown. Percentages of CD4+CD8\text{low} subpopulations are indicated.}
expression of active p56lck in the thymus interferes with the generation of normal levels of CD2. However, induced reduction of CD2 antigens occurs only in normal thymocytes but not in thymocytes lacking endogenous Rho function, p56lck downregulation of CD2 is thus a Rho-dependent response.

Discussion

The differentiation and proliferative expansion of pre-T cells is mediated via signaling pathways which involve activation of the tyrosine kinase p56lck (10, 11). However, nothing is known about other signaling molecules that operate with p56lck to mediate the complex molecular changes that occur at this stage of thymocyte development. To examine the role of the GTPase Rho in p56lck-mediated signals in the thymus, we generated mice double transgenic for active p56lck and the Rho inhibitor C3 transferase. This enabled us to compare thymocyte development in mice expressing active p56lck on a wild-type or Rho-2 background.

Mice with thymocyte-specific expression of a transgene encoding active p56lck show strong potentiation of proliferative responses in pre-T cells that results in a fivefold increase in numbers of CD442CD252 late pre-T cells. This p56lck-mediated response is abrogated when the active p56lck transgene is expressed in thymocytes which lack endogenous Rho function. Rho function is thus required for p56lck to drive the differentiation of pre-T cells from the CD442CD251 into the CD442CD252 compartment and to accelerate proliferative responses in this population. It has been shown that expression of active p56lck inhibits TCR-β chain rearrangements; DP thymocytes that develop in pLGF thymocytes fail to normally express αβ-TCR complexes (19). We now show that active p56lck can prevent expression of αβ-TCR complexes on DP thymocytes in the absence of endogenous Rho function. Interestingly, active p56lck also generates DP thymocytes that express downregulated levels of CD2 antigens when compared with normal thymocytes. However, our data show that p56lck does not prevent expression of CD2.
when expressed on a Rho- background. p56lck thus regulates thymocyte development at the pre-T cell stage by both Rho-dependent and Rho-independent responses.

It has been suggested that one critical role for p56lck at the pre-T cell stage of thymocyte development is to mediate allelic exclusion at the TCR-β chain locus (19, 26, 27). Expression of a constitutive active form of p56lck can initiate thymocyte differentiation in RAG-/- or pTα-/- mice (10, 11) and switch off rearrangements at the TCR-β locus. Further evidence for the role of p56lck in preventing rearrangement at the TCR-β locus stems from observations that DP thymocytes that develop in the presence of active p56lck fail to express TCR-β subunits. They do not show rearrangements at the TCR-β locus, do not express a functional α/β-TCR complex, and fail to develop into mature CD4+ or CD8+ SP thymocytes (19, 26).

The current model to explain this phenomenon is that p56lck acts as a sensor for the expression of a functional pre-TCR complex. Successful expression of a functional pre-TCR complex would normally activate p56lck and initiate the feedback mechanism that prevents further rearrangements of TCR-β chains, i.e., p56lck mediates allelic exclusion at the pre-T cell stage. The failure of TCR-β chain rearrangements in cells expressing active p56lck is thus thought to reflect the fact that such cells initiate the allelic exclusion mechanism before they have produced a competent TCR-β subunit. Once such cells differentiate into DP thymocytes there is no TCR-β chain to partner newly synthesized TCR-α subunits and consequently expression of a mature TCR-CD3 antigen receptor complex does not occur. This results in failed positive and negative selection and impaired development of mature SP thymocytes. The present data show that loss of Rho function has no influence on the ability of active p56lck to prevent expression of α/β-TCR complexes. Since the failure to express α/β-TCR complexes in thymocytes expressing active p56lck is a model for allelic exclusion, the simplest interpretation of the present data is that Rho function is not required for allelic exclusion at the TCR-β locus.

In this study, we demonstrate that expression of active p56lck in thymocytes not only prevents expression of mature α/β-TCR complexes but also regulates expression of the accessory molecule CD2. The downregulation of CD2 expression on pre-T cells is normally accompanied by up-regulation of the expression of the CD2 antigen (24, 25). Active p56lck can push the differentiation processes that downregulate expression of CD2, but the resultant cells do not express normal levels of CD2. The negative regulatory effect of active p56lck on CD2 expression can be reversed by a concomitant loss of Rho function. This was a particularly striking result because loss of Rho function did not restore expression of the α/β-TCR complex on pLGF thymocytes. The ability of active p56lck to prevent CD2 antigen expression on DP thymocytes is thus dependent on Rho function whereas the ability of p56lck to inhibit expression of the α/β-TCR complex is not.

In pLGF/C3 double transgenic mice we observed a consistent increase in frequencies of CD4-CD8low cells when compared with pLGF single transgenic mice. These ISP represent an intermediate stage in the transition from the DN to the DP compartment (21, 22). This result suggests that the transition of late pre-T cells into DP cells is partially suppressed in thymocytes lacking endogenous Rho function. However, loss of Rho function does not completely suppress pre-T cell differentiation. Moreover, although it strikingly decreases proliferative responses, it does not completely abrogate proliferation in late pre-T cells. The present results thus demonstrate that Rho is a regulator of pre-T cell development but they equally illustrate that compensatory, Rho-independent proliferative and differentiation signals must operate in early thymic progenitors. There is increasing awareness that pre-T cell differentiation is controlled by overlapping and compensatory signaling pathways. For example, the tyrosine kinases ZAP70 and Syk have overlapping and compensatory roles in pre-T cells (7) as do the transcription factors Tcf and Lef (23).

The present data show that Rho has an important function in p56lck-mediated signals for thymocyte development. The fact that pre-T cell differentiation is not absolutely dependent on Rho function is consistent with the fact that the thymic developmental block in mice deficient for p56lck is not absolute. In p56lck-deficient mice, the related kinase p59fyn can partially compensate for p56lck-mediated signals. Thymocytes that are deficient for both p56lck and p59fyn show a complete block in thymocyte development at the pre-T cell stage analogous to the developmental block seen in RAG-/- thymocytes (28). Therefore, one

![Figure 5](image-url)

**Figure 5.** Possible models for the positioning of Rho in p56lck-mediated signaling pathways regulating various aspects of pre-T cell development. (A) The pre-TCR initiates signaling pathways mediated by p56lck that regulate thymocyte development and control the process of allelic exclusion of the TCR-β locus in pre-T cells. A possible model consistent with our data locates Rho either directly downstream of p56lck or in parallel signaling pathways controlling proliferation and regulation of CD2 expression in pre-T cells. Other signals emerging from p56lck-regulating inhibition of TCR-β chain rearrangement are Rho independent. Dashed arrows symbolize as yet formally unproven links between the pre-TCR, p56lck, and Rho. (B) An alternative model compatible with our results depicts Rho as a signal amplifier which increases the signal intensity generated by p56lck. Individual biological responses regulated by the pre-TCR would be dependent on different signal intensities and would only be initiated if the signaling strength generated by p56lck and Rho would reach their individual thresholds. Thus, signal intensity in the absence of Rho function would still be sufficient to mediate inhibition of TCR-β chain rearrangement but not to downregulate CD2 expression or drive pre-T cell proliferation.
speculation is that loss of Rho function causes defects in p56lck-mediated function in pre-T cell but does not impact compensatory p59fyn signaling pathways. In this respect, the strategy used in this study to inactivate Rho function is extremely selective. C3 transferase has no inhibitory effects on the function of other Rho family GTPases such as Rho a-1, Rho a-2, or Cdc42 (29). It will be intriguing to know if loss of Rho function in the thymus can be compensated by the actions of these other Rho family GTPases.

In summary, the tyrosine kinase p56lck regulates pre-T cell proliferation and differentiation and controls expression of α/β-TCR complexes and CD2 antigens. This study shows that these p56lck-mediated responses can be distinguished by their requirement for Rho function. This requirement of Rho function for p56lck-mediated regulation of pre-T cell development is consistent with a model where Rho is part of signaling pathways that emerge from p56lck and regulate cell cycle progression and CD2 expression at this developmental stage. The signaling pathways emerging from p56lck that prevent expression of α/β-TCR complexes by suppressing TCR gene rearrangement at the β-locus are apparently independent of the function of Rho. One interpretation of these results is that qualitatively different signaling pathways, some involving Rho and some not, are being used to control these different p56lck responses (Fig. 5a). However, an alternative possibility is that Rho is regulating p56lck-signaling responses quantitatively. In this model, the same p56lck-induced signaling pathways controls thymocyte proliferation, α/β-TCR and CD2 expression but these responses require quantitatively different signaling thresholds (Fig. 5b). Rho could thus regulate the strength of p56lck-induced signals. This model predicts that regulation of TCR-β chain rearrangements has a low signaling threshold compared with the thresholds required for proliferation and control of CD2 antigen expression. Future studies will determine whether Rho is positioned directly downstream of p56lck at the pre-T cell stage or whether Rho is part of a parallel signaling pathway required for pre-T cell development.

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