The GTPase Rho has a critical regulatory role in thymus development

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The present study employs a genetic approach to explore the role of Rho GTPases in murine thymic development. Inactivation of Rho function in the thymus was achieved by thymic targeting of a transgene encoding C3 transferase from *Clostridium botulinum* which selectively ADP-ribosylates Rho within its effector domain and thereby abolishes its biological function. Thymi lacking functional Rho isolated from C3 transgenic mice were strikingly smaller and showed a marked (90%) decrease in cellularity compared with their normal litter mates. We also observed a similar decrease in levels of peripheral T cells in C3 transgenic mice. Analysis of the maturation status of thymocytes indicated that differentiation of progenitor cells to mature T cells can occur in the absence of Rho function, and both positive and negative selection of T cells appear to be intact. However, transgenic mice that lack Rho function in the thymus show maturational, proliferative and cell survival defects during T-cell development that severely impair the generation of normal numbers of thymocytes and mature peripheral T cells. The present study thus identifies a role for Rho-dependent signalling pathways in thymocyte development. The data show that the function of Rho GTPases is critical for the proliferative expansion of thymocytes. This defines a selective role for the GTPase Rho in early thymic development as a critical integrator of proliferation and cell survival signals.
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Introduction

T-cell development within the thymus involves an ordered sequence of differentiation and proliferation events. Bone marrow-derived prothymocytes which enter the thymus undergo phases of proliferative expansion, T-cell antigen receptor (TCR) rearrangements and positive and negative selection processes to mature into peripheral T cells. These thymic developmental events are essential to ensure that sufficient cells with the appropriate immune repertoire exit to the periphery. Individual stages of thymic differenti-

ation can be distinguished phenotypically on the basis of CD4 and CD8 co-receptor expression: immature CD4[−]CD8[−] double negative (DN) cells make the transition to mature CD4⁺ or CD8⁺ single positive (SP) cells via an intermediate CD4⁺CD8⁺ double positive (DP) stage. The proliferative expansion phases of thymocyte development are regulated by members of the haematopoietin family of cytokines, particularly interleukin-7 (IL-7) (Peschon *et al.*, 1994; Cao *et al.*, 1995; DiSanto *et al.*, 1995; Nosaka *et al.*, 1995; Russell *et al.*, 1995; Thomis *et al.*, 1995; von Freeden-Jeffry *et al.*, 1995). One critical differentiation point in the thymus, the transition of T cells from the CD4[−]CD8[−] DN stage into DP cells is regulated by a signalling complex designated the pre-TCR complex (Fehling *et al.*, 1995). This comprises the TCR β chain, the pre-TCR α -chain and CD3 antigen subunits (Groettrup and von Boehmer, 1993; Groettrup *et al.*, 1993). A second crucial checkpoint, the differentiation of CD4⁺CD8⁺ DP to mature CD4⁺ or CD8⁺ SP thymocytes is controlled by a functional $\alpha\beta$ TCR–CD3 antigen complex. This stage of thymic differentiation involves a selection process which allows only those cells bearing a receptor capable of recognizing 'foreign' antigen in context with 'self' class I or class II major histocompatibility complex (MHC) molecules to pass to the peripheral lymphoid circulation (von Boehmer, 1992). Antigen receptor occupancy in DP thymocytes yields two very different outcomes: inappropriate binding of the TCR to peptide–MHC induces cell deletion (negative selection) (Kisielow *et al.*, 1988; Iwabuchi *et al.*, 1992), whereas those thymocytes that interact with peptide and MHC correctly are positively selected for survival and further differentiation (von Boehmer, 1992; Hogquist *et al.*, 1993).

Lymphocyte development in the thymus is dependent on signalling pathways generated by a complex array of extracellular stimuli involving antigen receptor- and cytokine receptor-derived signals (Owen and Venkitaraman, 1996). These signalling events are required to regulate and order such processes as antigen receptor gene rearrangement, gene expression, cell death and cell proliferation. Recently, there has been some progress in the identification of key intracellular signalling molecules which are central to the coordination of thymic development. One intracellular signalling molecule that regulates proliferative expansion of thymocytes is the Janus kinase JAK3, a tyrosine kinase associated with the common γ -chain of the IL-7R (Nosaka *et al.*, 1995; Park *et al.*, 1995; Russell *et al.*, 1995; Thomis *et al.*, 1995). The tyrosine kinases p56^{lck}, ZAP70 and ITK are also essential for mediating pre-TCR and $\alpha\beta$ TCR functions in the thymus (Levin *et al.*, 1993; Anderson *et al.*, 1994; Charlene-Liao and Littman, 1995; Negishi *et al.*, 1995; Wallace *et al.*, 1995).

Tyrosine kinases act proximal to the receptor to initiate downstream signalling cascades. Much less is known about the molecular switches that translate signals from these tyrosine kinases. One major advance has been the recognition from several elegant studies that the GTPase Ras plays a role in the regulation of thymocyte differentiation (Alberola-Ila *et al.*, 1995, 1996; Swan *et al.*, 1995; Swat *et al.*, 1996). Hence Ras and the ERK2 kinase MEK, which is a target for Ras signals in T cells, can operate at the two major differentiation points in the thymus: signalling pathways controlled by the GTPase Ras participate in the thymic differentiation pathways induced by the pre-TCR and selection processes governed by the mature TCR (Alberola-Ila *et al.*, 1995, 1996; Swan *et al.*, 1995; Crompton *et al.*, 1996; Swat *et al.*, 1996). Strikingly, Ras signalling pathways play a selective role in determining thymocyte fate: the Ras/Raf-1/MEK pathway is thus required for the positive selection of mature SP thymocytes but is not involved in the signalling processes that control the negative selection of thymocytes (Alberola-Ila *et al.*, 1995, 1996; Swan *et al.*, 1995; O'Shea *et al.*, 1996). The Ras/MEK pathway also seems to have a minimal role in regulating the proliferative expansion phases of thymocyte development (Alberola-Ila *et al.*, 1995; Crompton *et al.*, 1996).

Recent studies have suggested that GTPases of the Rho family of proteins may have important functions in haematopoietic cells: the Wiscott–Aldrich immunodeficiency syndrome has been mapped to a defect in an effector protein for the Rho family GTPase Cdc42 (Crespo *et al.*, 1996). Mice deficient for Vav, a putative guanine nucleotide exchange factor (GEF) for multiple GTPases of the Rho family, show defects in T- and B-cell biology (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995; Crespo *et al.*, 1996; Olson *et al.*, 1996). Finally, the GTPase Rho has been implicated in signalling processes in leucocytes including integrin signal transduction and cell-mediated cytotoxicity (Lang *et al.*, 1992; Laudanna *et al.*, 1996). There is also considerable evidence that Rho-derived signals are important in the initiation of transcription factor responses (Hill *et al.*, 1995) and cell cycle control mechanisms in fibroblasts (Olson *et al.*, 1995), although the role of Rho in regulating this type of cellular response in lymphocytes has not been examined. In the present study, we have taken a genetic approach to examine the role of Rho in thymocyte development. Inactivation of Rho function in the thymus was achieved by thymic targeting of a transgene encoding bacterial C3 transferase from *Clostridium botulinum* which selectively ADP-ribosylates Rho and thereby abolishes its biological function (Lang *et al.*, 1992; Ridley and Hall, 1992; Yamamoto *et al.*, 1993; Aktories and Just, 1995b; Hill *et al.*, 1995). Differentiation of thymocytes to mature T cells can occur in the absence of Rho function, and both positive and negative selection of thymocytes appear to be intact. However, transgenic mice that lack Rho function in the thymus show a striking proliferative and cell survival defect during T-cell development that severely impairs the generation of normal numbers of thymocytes and mature peripheral T cells. The present study thus defines a selective role for Rho in thymic development as a critical determinant of proliferation and cell survival signals.

Results

Functional inactivation of Rho by thymic expression of a transgene encoding bacterial C3 transferase from *C.botulinum*

To block Rho function in thymocytes, we used a well-established transgenic expression vector based on the proximal promoter of p56^{lck} (Garvin *et al.*, 1988; Chaffin *et al.*, 1990; Anderson *et al.*, 1993; Levin *et al.*, 1993; Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995; O'Shea *et al.*, 1996) to target expression of *C.botulinum* C3 transferase to the thymus of transgenic mice (Figure 1A). C3 transferase specifically ADP-ribosylates Rho (Aktories and Just, 1995a; Lang and Bertoglio, 1995) at one site (Asn41) in its effector domain, thereby inhibiting its cellular function (Ridley and Hall, 1992; Hill *et al.*, 1995). The selectivity of C3 transferase for Rho has been documented extensively in both lymphoid cells and fibroblasts (Hill *et al.*, 1995; Lang and Bertoglio, 1995). Four transgene-carrying mice were identified by PCR-based screening and were backcrossed to C75/BL6 inbred mice to generate founder lines. Transgene expression was determined in all founder lines by RT-PCR from thymic mRNA (Figure 1B). In ADP-ribosylation assays using [³²P]NAD and recombinant C3, only thymocyte lysates from normal litter control mice yielded a 25 kDa *in vitro* [³²P]ADP-ribosylated band corresponding to Rho (Figure 1C, lane 1). In contrast, thymocyte lysates from C3 transgenic mice did not show any significant [³²P]ADP-ribosylation *in vitro*, indicative of an already completely ribosylated status of endogenous Rho and confirming the functional inactivation of Rho in thymocytes of C3 transgenic mice (Figure 1C, lane 2).

Thymi from C3 transgenic mice (Rho⁻ thymi) were strikingly smaller (Figure 2A) and showed a marked decrease in cellularity (5–20%) compared with their normal litter mates. (Figure 2C, left panel). Histological sections of these small thymi showed a severe disruption of normal thymic morphology with an unclear cortico-medullary junction (Figure 2B). This phenotype was similarly observed in all established founder lines. Peripheral lymphoid organs such as spleen and mesenteric lymph nodes did not show the striking reduction in size observed in transgenic thymi. However, cellularity in spleens of C3 transgenic mice was only 50% of normal (Figure 2C, right panel). Further examination of the cellular composition of total spleen populations using antibodies reactive with B- or T-cell markers revealed that the marked reduction of cellularity was accounted for mainly by a severe drop in levels of peripheral αβTCR-positive T cells (Figure 3A). The low levels of peripheral T cells in transgenic mice were, however, apparently normal with respect to expression of the TCR and comprised the normal ratio of CD4⁺ or CD8⁺ T-cell subsets (data not shown).

Frequencies of CD4/CD8 thymocyte subsets are changed in Rho⁻ thymocytes

The loss of thymus cellularity can result from a block in thymocyte differentiation prior to the main point of proliferative expansion as cells acquire CD4 and CD8 expression and switch from CD4⁻ DN to CD4⁺8⁺ DP phenotype. To explore the differentiation status and hence the mechanisms underlying the loss of cellularity in the

Fig. 1. Generation of *lck*-C3 transgenic mice. (A) Diagram of the *lck*-C3 transgene. The transgenic vector p1017 (Chaffin *et al.*, 1990) consists of a pUC19 backbone and a 3.2 kb fragment of the mouse *lck* gene that contains the proximal promoter juxtaposed to a human growth hormone (hGH) genomic sequence (alternating filled and hatched boxes represent exon and intron sequences). A 0.9 kb cDNA encoding C3 transferase from *C. botulinum* was subcloned into the *Bam*HI cloning site of p1017, positioning the transgene immediately downstream of the *lck* promoter into the first exon of the hGH gene. Arrows indicate the position of transgene-specific primer pairs used in PCR-based screening for transgene-carrying mice. (B) C3 mRNA is expressed in thymocytes of C3 transgenic mice. Four transgene-carrying founder mice were identified by PCR-based screening and backcrossed to C75/BL6 inbred mice to generate founder lines. Transgene expression was determined in all founder lines by RT-PCR from thymic mRNA. Results from RT-PCR of thymocyte mRNA employing transgene- and β -actin-specific primer pairs are shown for normal litter control mice (lanes 1–4) and transgenic mice (lanes 5–8) from founder line 2A (lanes 1 and 5), 3D (lanes 2 and 6), 2D (lanes 3 and 7) and 2C (lanes 4 and 8). (C) Rho from C3 transgenic thymocytes is no longer a substrate for *in vitro* ADP-ribosylation by recombinant C3. Thymocytes (4×10^6) from normal litter control (lane 1) and C3 transgenic mice (lane 2) were homogenized and subjected to ultracentrifugation. The 1000 g supernatant was subjected to *in vitro* ADP-ribosylation employing [32 P]NAD as described (Aktories *et al.*, 1992). ADP-ribosylated samples were separated by SDS-PAGE and visualized by autoradiography.

Rho⁻ thymus, we analysed the CD4/CD8 phenotype of thymocytes from C3 transgenic mice by flow cytometry. As shown in Figure 3B, all normal CD4/CD8 subsets were present in Rho⁻ thymocytes. The observed drop in total thymic cellularity reflected a reduction in cell numbers of all subsets (Table I), in particular a striking reduction in the percentage and numbers of CD4⁺8⁺ DP thymocytes. Examination of $\alpha\beta$ TCR expression in all four CD4/CD8 thymocyte subsets using an anti β -chain monoclonal antibody (mAb) revealed no significant differences in TCR levels between normal control and Rho⁻ thymocytes (Figure 3C). These data show that mature SP thymocytes can develop, although at a reduced rate, in the absence of Rho function.

Characterization of CD4⁺8⁻ DN thymocyte subpopulations in Rho⁻ thymocytes

To examine thymocyte subpopulations in Rho⁻ thymi further, we performed a detailed phenotypic analysis of thymic precursor cells. Immature DN thymocytes have been subdivided into four distinct phenotypic precursor populations by the differential expression of CD25 and CD44, providing a maturational sequence of CD25⁻CD44⁺ → CD25⁺CD44⁺ → CD25⁺CD44⁻ → CD25⁻CD44⁻ cells (Pearse *et al.*, 1989; Godfrey and Zlotnik, 1993; Godfrey *et al.*, 1993). We therefore studied CD25/CD44 expression of CD4⁺8⁻ DN thymocytes gating on cells negative for CD4, CD8, CD3, B200, Mac-1 and Gr-1 surface markers, thereby excluding mature T cells, B

cells, macrophages and granulocytes from this analysis. Compared with normal controls, CD4⁺8⁻ DN thymocytes lacking Rho function contain a low percentage of CD25⁺ cells concomitant with an increased frequency of CD25⁻ thymocytes, both of the CD44⁺ and CD44⁻ phenotype (Figure 4A). Calculations of absolute cell numbers in the Rho⁻ thymus revealed a severe reduction in CD25⁺ cells and also a reduction in CD25⁻CD44⁻ cells. In contrast, we observed an unchanged number of immature CD25⁻CD44⁺ DN cells (Table II). Several markers besides CD44 and CD25 have been used to characterize DN thymocyte subsets further, these include Thy-1, heat-stable antigen (HSA) and the receptor for steel-factor, c-Kit (Godfrey *et al.*, 1992). The data in the upper panel of Figure 4B show that expression profiles for HSA (CD24), c-Kit (CD117) and Thy-1 (CD90) of CD44⁺ DN cells from Rho⁻ thymi closely resemble profiles from normal CD44⁺ DN thymocytes, in agreement with the described phenotype of early thymic precursor cells (Godfrey *et al.*, 1993). The maturational status of CD4⁺8⁻ DN precursor cells in C3 transgenic mice was also examined in the context of expression of CD2. Since late DN cells express CD2 before acquisition of CD4 and CD8 (Rodewald *et al.*, 1993; Lalli *et al.*, 1996), we analysed the phenotype of CD4⁺8⁻ DN cells by studying CD25 and CD2 expression of thymocyte precursors of control and C3 transgenic mice. As shown in Figure 4C, about half of the CD4⁺8⁻ DN precursors from control mice are CD25⁻ and these are somewhat equally distributed between CD25⁻CD2⁻

Fig. 2. Size, morphology and cellularity of the thymus and spleen isolated from C3 transgenic mice. (A) Shown is a freshly prepared thymus from a 5-week-old wild-type (left) and a representative age-matched C3 transgenic (right) mouse at the same magnification (1:10). (B) Morphology of a thymus from a 5-week-old wild-type (left) and a representative age-matched C3 transgenic (right) mouse. Shown is a standard histological staining with haematoxylin and eosin (magnification 1:50). (C) Cell numbers of thymus (left panel) and spleen (right panel) of 5- to 8-week-old C3 transgenic (●) and normal litter control mice (○). Bars indicate means from $n = 12$ and $n = 10$ mice, respectively.

Fig. 3. Phenotypal analysis of thymocytes and peripheral T-cell population in Rho^{-} mice. **(A)** Splenocytes from 6-week-old normal control mice (left) and C3 transgenic mice (right) stained for T-cell (anti-TCR β , H57-5970) and B-cell (anti-B220, CD45R, RA3-6B2) surface markers. The percentages of populations falling into individual quadrants are indicated. **(B)** C3 thymocytes comprise a low percentage of DP cells but an increased frequency of DN and SP cells. Representative dual parameter histograms for CD4 (PE, RM4-5) and CD8 (FITC, 53-5.8) expression of total thymocytes isolated from normal control (left) and age-matched C3 transgenic mice (right). The percentages of individual CD4/CD8 subpopulations are indicated. Data shown are representative of five experiments. **(C)** C3 transgenic mice show normal TCR expression levels in all CD4/CD8 thymocyte subsets. Histograms show expression of $\alpha\beta$ TCR in CD4/CD8 thymocyte subsets of normal control (upper panel) and age-matched C3 transgenic mice (lower panel). Total thymocytes were stained for CD4 (PE, RM4-5), CD8 (biotinylated, 53-5.8), $\alpha\beta$ TCR (β -chain, FITC, H57-597) and $\alpha\beta$ TCR expression gated on the indicated CD4/CD8 subpopulations.

Table I. Cell numbers of thymocyte subsets in C3 transgenic and normal control mice

Population	Control	C3 transgenic
Total	158.0 $\pm$ 45	18.0 $\pm$ 10
CD4 ⁻ CD8 ⁻	6.3 $\pm$ 1.7	1.8 $\pm$ 0.9
CD4 ⁺ CD8 ⁺	124.7 $\pm$ 36.9	10.8 $\pm$ 7.5
CD4 ⁺ CD8 ⁻	15.7 $\pm$ 2.9	3.7 $\pm$ 2.1
CD4 ⁻ CD8 ⁺	7.9 $\pm$ 1.7	1.8 $\pm$ 1.0

Total cell numbers were determined for $n = 12$ and $n = 10$ mice at 5–8 weeks of age. Phenotypes were determined by two-colour flow cytometry employing anti-CD4 and anti-CD8 mAbs, and values for individual subpopulations were calculated on the basis of percentages obtained. (mean $\pm$ SD) (cell number $\times 10^6$).

and CD25⁻CD2⁺ compartments. As already observed in the CD25/CD44 analysis, ~90% of CD4⁻8⁻ DN cells from C3 transgenic mice were CD25⁻; there was, however, no distinctive accumulation in either the CD25⁻CD2⁻ (immature) or CD25⁻CD2⁺ (mature) compartment (Figure 4C). We conclude, therefore, that thymocyte precursor maturation in C3 transgenic mice leads to genuine differen-

tiated progenitor cells with a typical CD44⁻CD25⁻CD2⁺ phenotype.

Cell cycle and apoptosis analysis of Rho^{-} thymocytes

The major proliferative expansion of thymocytes occurs in two waves within the DN compartment. Progenitor cells undergo a phase of expansion before rearrangement of the TCR β genes, and pre-TCR-expressing mature DN cells differentiate and proliferate to generate DP thymocytes. To explore the mechanism underlying the decrease in thymocyte cell numbers, we analysed the cell cycle status of DN thymocytes isolated from C3 and control mice. Freshly isolated thymocytes were surface stained for CD4 and CD8, and the DNA content of CD4⁻8⁻ DN cells was analysed by staining with propidium iodide. CD4⁻8⁻ DN thymocytes lacking Rho function show a relative increase of cells in the G₁ phase of the mitotic cell cycle and a severe reduction of cells in S and G₂ + M phases (Figure 5A), indicating a partial G₁ block of DN thymocytes in C3 transgenic mice. There are a series of checkpoints in the life cycle of mammalian cells,

Fig. 4. Analysis of thymic precursor populations in C3-transgenic mice. **(A)** Thymic progenitor cells from C3 transgenic mice contain low frequencies of CD25⁺ cells. Thymocytes were stained for expression of CD25 (FITC), CD44 (PE) and a panel of mature lineage markers including CD4, CD8, CD3, B220, Gr-1 and Mac-1 (all biotinylated) and analysed by flow cytometry. Cells lacking expression of the mature lineage markers were gated and then examined for CD25 and CD44 expression. Representative dual parameter dot-plots of age-matched control and C3 transgenic mice are shown, and the frequencies of individual gated populations are indicated. **(B)** CD44⁺ thymic progenitor cells from C3 transgenic mice show normal surface expression of HSA, c-Kit and Thy-1. Total thymocytes were stained for expression of CD44 (PE), a panel of mature lineage markers including CD4, CD8, CD3, B220, Gr-1 and Mac-1 (all biotinylated) and either HSA (FITC), c-Kit (FITC) or Thy-1 (FITC) and analysed by flow cytometry. Cells negative for all mature lineage markers and positive for CD44 were gated and then examined for HSA, c-Kit or Thy-1 expression, respectively. Representative histograms of age-matched control (grey shaded) and C3 transgenic (solid black line) mice are shown. The bottom panel shows the lack of a blastoid cell population in CD25⁺CD44⁺ DN thymocytes from Rho^{-/-} thymi. Thymocytes were stained for expression of CD25 (FITC), CD44 (PE) and a panel of mature lineage markers as above and analysed by flow cytometry. Cells lacking expression of the mature lineage markers and both CD25 and CD44 markers were gated and analysed for forward light scatter profiles. Representative histograms of an age-matched control (grey shaded profile) and C3 transgenic (solid black line) mouse are shown. **(C)** CD25⁺ thymic progenitor cells from C3 transgenic mice contain both CD2⁺ and CD2⁻ cells. Thymocytes were stained for expression of CD25 (FITC), CD2 (PE) and a panel of mature lineage markers as above and analysed by flow cytometry. Cells lacking expression of the mature lineage markers were gated and examined for CD25 and CD2 expression. Representative dual parameter dot-plots of age-matched control and C3 transgenic mice are shown, and the frequencies of individual gated populations are indicated.

and cells that fail to traverse these are programmed to die by apoptosis. Analysis of apoptosis in *ex vivo* thymocytes by double staining with propidium iodide and Hoechst 33342 revealed a marked increase in cell death in Rho^{-/-} thymocytes compared with normal controls (Figure 5B).

Our data in Figure 4A and Table II reveal a loss of cellularity in the CD25⁺CD44⁺ late stage DN thymocytes from Rho^{-/-} thymi. This late stage DN population should be in cycle and contain a high percentage of blastoid cells. We were, however, unable to detect any cycling cells in

the CD25⁻CD44⁻ population. Moreover, the data in the lower panel of Figure 4B show that this population does not contain cells with an increased forward light scatter profile, indicating a lack of blastoid thymocytes in this compartment.

Table II. Cell numbers of CD4⁻8⁻3⁻ triple negative thymocytes subsets in C3 transgenic and normal control mice

Population	Control	C3 transgenic
Total	61.1 ± 16.0	8.6 ± 4.3
CD25 ⁻ CD44 ⁺	1.8 ± 1.2	1.8 ± 0.6
CD25 ⁺	32.3 ± 5.3	0.7 ± 0.3
CD25 ⁻ CD44 ⁻	25.6 ± 5.1	5.3 ± 1.5

CD25/CD44 expression of CD4⁻8⁻3⁻ triple negative thymocytes was determined by three-colour flow cytometry excluding cells positive for CD4, CD8, CD3, B220, Mac-1 and Gr-1. Absolute cell numbers in individual CD25/CD44 subpopulations were calculated on the basis of percentages obtained and average numbers of CD4⁻8⁻3⁻B220⁻Mac-1⁻Gr-1⁻ thymocytes in control and C3 transgenic mice. (mean ± SD) (cell number × 10⁵).

Rho function is not required for positive and negative selection of thymocytes

Differentiation of DP to mature CD4⁺ or CD8⁺ SP thymocytes requires the positive selection of cells with an appropriate receptor repertoire as well as the deletion of cells with the potential for self-reactivity (von Boehmer, 1992; Owen and Venkitaraman, 1996). The presence of apparently normal populations of mature αβTCR^{high} CD4⁺ or CD8⁺ SP thymocytes suggests that these processes occur in Rho⁻ mice (Figure 3). However, to examine directly the role of Rho in positive and negative selection, we analysed thymocytes from mice made doubly transgenic for C3 transferase and a TCR specific for the male HY antigen presented by H-2 D^b molecules (Kisielow *et al.*, 1988; Swat *et al.*, 1991). Thymocytes expressing the HY-specific TCR are negatively selected in male mice and undergo massive deletion resulting in few detectable DP or SP thymocytes. This process was not affected by co-expression of the C3 transferase (Figure 6A). Thymocytes from female mice transgenic for the HY-specific TCR are positively selected and differentiate into

Fig. 5. Cell cycle and apoptotic status of Rho⁻ thymocytes. (A) Flow cytometric analysis of cellular DNA content by propidium iodide (PI). Data show PI-stained permeabilized DN thymocytes from C3 transgenic mice (right) compared with normal control mice (left). Total thymocytes were prepared as described and stained with anti-CD8 and anti-CD4 (both FITC) mAbs, fixed and DNA stained with PI. Histograms show PI profiles gated on thymocytes lacking CD4 and CD8 expression. The percentages of cells falling within markers representing individual stages of the cell cycle were: G₁, 78 and 96%; S, 20 and 4%; G₂ + M, 3 and 0.1% (for control and C3 transgenic thymocytes, respectively). (B) Dual parameter flow cytometric analysis of freshly isolated thymocytes from C3 transgenic mice (right) compared with normal control mice (left) stained for early (Hoechst 33342-positive) and late (Hoechst 33342/propidium iodide-positive) apoptotic cells. The percentages of cells positive for both dyes are indicated.

Fig. 6. Analysis of positive and negative selection processes in Rho⁻ thymocytes. Thymocytes from male (A) or female (B) F1 progeny of HY-TCR $\alpha\beta$ homozygous mice with (right panel) or without (left panel) C3 transgene expression were compared. Thymocytes were prepared as described and stained with anti-CD8 (FITC), anti-CD4 (PE) and anti-HY-TCR α (T3.70, biotinylated) mAbs, as described, and analysed by flow cytometry. Panels show CD4/CD8 profiles gated on T3.70^{hi} thymocytes. The analysis shown is representative of five experiments with similar results.

mature CD8⁺ SP thymocytes. Female HY-TCR/C3 double transgenic mice also showed normal proportions of mature CD8⁺ SP thymocytes in comparison with HY-TCR transgenic litter mates (Figure 6B). However, an ~10-fold reduction in total cell recovery compared with HY-TCR litter mates was noted in both male and female HY-TCR/C3 double transgenic mice. The introduction of a transgenic TCR could not rescue the proliferative defect of Rho⁻ thymocytes, indicating that the cause of this phenotype cannot be related to an improper antigen receptor gene rearrangement. The transgenic TCR was expressed at equivalent levels in the majority of thymocytes in both female HY-TCR transgenic and HY-TCR/C3 double transgenic mice (not shown). These findings indicate that blockage of Rho function has no discernible effect on positive and negative selection processes in the thymus.

The apparent normality of thymocyte selection and the mature phenotype of SP thymocytes in C3 transgenic mice prompted us to explore the *in vitro* functional capabilities of mature SP Rho⁻ thymocytes in response to TCR stimulation. CD4⁺ SP thymocytes were isolated and cultured *in vitro* with CD3 antibodies in the presence or absence of IL-2. Rho⁻ thymocytes were able to respond to these typical conditions of *in vitro* activation as judged using [³H]thymidine incorporation as a marker of cell cycle entry (Figure 7). There were no discernible differences in these *in vitro* activation responses between normal and Rho⁻ thymi.

Fig. 7. TCR-stimulated *in vitro* proliferation of mature CD4⁺8⁻ Rho⁻ thymocytes. Total thymocytes from control and C3 transgenic mice were prepared as described and stained with anti-CD8 (FITC) and anti-CD4 (PE) mAbs. CD4⁺8⁻ SP thymocytes were isolated by cell sorting and stimulated with anti-CD3 mAb alone or in the presence of IL-2. Bars show means of duplicates, the experiment shown is representative of three such experiments carried out independently.

Discussion

The present data show that function of the GTPase Rho is essential for normal thymic development, thus defining that Rho has a critical intracellular signalling role in T lymphocytes. Subpopulations of thymocytes representing all major stages in thymocyte development typically found in wild-type mice are also present in mice lacking thymic Rho function, but in severely reduced numbers. This indicates that thymocyte differentiation is able to proceed in the absence of Rho function. We could show that there was a decrease in the number of thymocytes traversing the cell cycle in Rho⁻ thymi and also increased levels of apoptotic cells. We conclude accordingly that the loss of cellularity in Rho⁻ thymi is due to a failure of thymocytes to progress through the G₁ phase of the cell cycle at the normal rate and a concomitant decreased survival capacity. These data demonstrate that Rho function is essential for the maintenance and proliferative expansion of thymocyte subsets. Further studies will be required to resolve the mechanism of action of Rho in the thymus and to explore the roles of the reported effector molecules for Rho such as Rho-binding kinase (ROK) (Leung *et al.*, 1996; Matsui *et al.*, 1996) or protein kinase N (PKN) (Amano *et al.*, 1996; Watanabe *et al.*, 1996) in T-cell development and activation. Rho signalling pathways are not yet well defined in any cell system, but the present data show that effectors of Rho must have a critical role in thymic development.

A major point of thymocyte proliferative expansion occurs in CD4⁺8⁻ DN cells and in cells that have made the commitment to transit to the CD4⁺8⁺ DP stage. To analyse the DN cells in Rho⁻ thymi more closely, CD4⁺8⁻ DN cells were examined for expression of CD44 and CD25 by flow cytometry. The dynamic expression of these markers defines distinct stages of DN differentiation and, during development, CD25⁺CD44⁺ cells give rise to CD25⁺CD44⁺ cells which then make the transition to a CD25⁺CD44⁻ stage. The present results show that lack of

Rho function causes a perturbation of the normal ratio of DN thymocyte subpopulations in thymi lacking functional Rho, with a prominent depletion of CD25⁺CD44⁺ cells in the Rho⁻ thymus. The acquisition of CD25 expression by CD25⁻CD44⁺ cells is an event that is accompanied by extensive proliferation. The loss of CD44⁺CD25⁺ cells could thus be explained by a failure of the proliferative expansion of the CD25⁺ population in the Rho⁻ thymus. There was also a loss in numbers of late stage DN cells (CD44⁺CD25⁺) in the Rho⁻ thymus. These losses could be caused by an incomplete/leaky maturation defect that inhibits CD44⁺CD25⁻ cells differentiating into the CD44⁺CD25⁺ stage and slows down the differentiation rate of the DN to DP transition. Thymocyte proliferative expansion also occurs once cells have made the commitment for DN to DP transition. Similarly, the reduced frequency of CD4⁺8⁺ DP cells in the Rho⁻ thymus is compatible with a model in which Rho signals are required for the proliferative expansion which normally occurs at this stage of thymocyte development.

The phenotype of the Rho⁻ thymus, particularly the subtle skewing of the thymocyte subpopulations, is consistent with inhibition of the major points of thymocyte proliferative expansion. The perturbations in cell cycle entry and the survival capacity of CD4⁺8⁻ DN cells also indicate that Rho mediates G₁ progression and cell survival signals initiated by receptors which deliver signals leading to thymocyte proliferation at early stages of thymopoiesis. Thymocyte proliferative expansion at the early stages of DN cell development, particularly of the CD25⁻CD44⁺ and CD25⁺CD44⁺ cells, is known to be regulated by the cytokine IL-7. In this context, the reduction in thymic cellularity caused by a loss of Rho function resembles that seen in mice lacking components of the IL-7 receptor signalling complex (Zeng *et al.*, 1994; Cao *et al.*, 1995; DiSanto *et al.*, 1995; Ihle, 1995; von Freeden-Jeffry *et al.*, 1995). Failure of thymic proliferative expansion *in vivo* but apparently normal differentiation of mature T cells was also observed in mice lacking the tyrosine kinase Jak3 which is activated by a variety of cytokine receptors that use the common IL-2R γ subunit (Cao *et al.*, 1995; Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995). These similarities in phenotype raise the issue that the GTPase Rho plays a role in IL-7R/IL-2R γ /Jak3 signal transduction pathways. However, the differentiation of DN thymocytes to DP cells and the proliferative expansion of the late stage DN cells is controlled by pre-TCR-driven signals (Crompton *et al.*, 1994; Fehling *et al.*, 1995). Pre-TCR-driven DN to DP thymocyte differentiation can still occur in Rho⁻ thymi but the reduced numbers of DP cells would indicate that a loss in Rho function compromises pre-TCR-initiated proliferative signals. Interestingly, the present results show that reduced numbers of mature SP T cells in the Rho⁻ thymi gave normal proliferative responses to *in vitro* stimulation of the mature TCR complex. The involvement of Rho-mediated signals in proliferative expansion of thymocyte progenitors *in vivo* but not mature SP thymocytes *in vitro* thus indicates that the signal transduction pathways that regulate proliferative responses in discrete T-cell populations differ. Moreover, responsiveness of Rho⁻ thymi to IL-2 *in vitro* indicates that Rho does not play a generic role in signal transduction

by members of the haematopoietin cytokine receptor family.

Mice lacking Vav, a putative GEF for multiple members of the Rho family of small GTPases (Crespo *et al.*, 1996), show reduced thymocyte cellularity (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995). Vav acts to regulate the activity of Rac, Cdc42 and Rho in fibroblasts (Crespo *et al.*, 1996; Olson *et al.*, 1996). Vav function is required for the proliferative expansion of thymocytes and, in this respect, the phenotype of the Vav^{-/-} thymocytes overlaps with the phenotype of the Rho-deficient thymi presented herein. Vav can activate Rho-dependent signalling responses in fibroblasts (Olson *et al.*, 1996), and defects in Rho responses could thus underlie the defects in Vav^{-/-} mice. However, Vav^{-/-} mice also show defects in TCR-mediated positive selection and *in vitro* activation responses of mature CD4 SP cells that were not observed in Rho⁻ thymocytes. This could be attributed to effects of Vav on Rac and Cdc42 (Olson *et al.*, 1996), which are not substrates for the enzymatic inactivation by C3 transferase. Also, Vav has an adapter protein function mediated by its SH2/SH3 domains in mature T cells which could explain the deleterious effects of Vav deficiency in these cells (Wu *et al.*, 1996).

Transgenes based on the p56^{lck} promoter have been used successfully to express inhibitory mutants of the GTPase Ras and the Ras effectors Raf1 and MEK in the thymus (Swan *et al.*, 1995; Alberola-Ila *et al.*, 1996; O'Shea *et al.*, 1996). The phenotype of thymi expressing these molecules is completely different from the phenotype of the Rho⁻ thymi described herein. Molecules that interfere with Ras function thus impair thymocyte differentiation and block the processes of positive selection and the generation of mature SP T cells (Alberola-Ila *et al.*, 1996; O'Shea *et al.*, 1996). Moreover, SP thymocytes expressing inhibitory Ras or Raf1 transgenes show *in vitro* activation defects in response to lectins or TCR agonists. These phenotypes were not seen in the Rho⁻ thymi, indicating that Ras and Rho operate independently during thymic differentiation. There was no major loss of thymocyte cellularity caused by expression of inhibitory Ras, Raf1 or MEK molecules analogous to that seen in the Rho⁻ thymus. Notably, MEK can mediate pre-TCR-driven differentiation but not proliferation signals (Crompton *et al.*, 1996). The present data establish that the GTPase Rho integrates a different and reciprocal facet of thymocyte development from that regulated by Ras. Thus Ras signals are required for thymocyte differentiation but not for the proliferative expansion of thymocyte progenitors *in vivo*. Conversely, Rho signals are crucial for thymocyte proliferation and cell survival but have no obligate role in thymocyte differentiation. The actions of Ras and Rho at discrete stages in thymocyte development show that members of the Ras superfamily of GTPases coordinate the complex signals that determine the T-cell repertoire.

Materials and methods

Transgenic mice

A purified 6.2 kb *NotI* fragment (2 ng/ μ l) containing the *lck*-C3-hGH transgene was injected into the pronuclei of day1 F1 mouse zygotes (C57/BL6/J $\times$ CBA/J). Embryos were transferred into pseudo-pregnant F1 foster mice which gave birth to litters of 5–10 mice 20 days later.

Stable lines were generated by backcrossing transgene-carrying founder mice with C57/BL6/J mice or HY-TCR transgenic mice.

PCR

Transgene-carrying mice were identified by PCR employing transgene-specific primers: p56S (GAGGGAACCCAGTCAGGAGC), C3NT (CTGATTGCTTAGTCCATAC), G9112 (GCCACCATGGAGCAGA-AGCTGATCTCCG), C3CT (GGGCACAGCTATCAATCC) and hGH (GCGCTTACCTGTAGCCATTGC). Genomic DNA was purified from mouse tail and used as template for 30 cycles of PCR (57°C annealing, 72°C elongation, 95°C denaturation).

RT-PCR

Total RNA was prepared by acid-phenol-chloroform extraction of guanidinium thiocyanate lysates from 5×10^6 cells (Chomczynski and Sacchi, 1987), DNase treated and subjected to reverse transcriptase reaction employing random hexamer primers. cDNA was used as template in transgene-specific PCR as described above.

Cell preparation

Thymi and spleens were obtained by dissection from 5- to 8-week old mice. Tissue was disaggregated by mincing with fine forceps and forced through a fine mesh filter to obtain a single cell suspension. Splenocytes additionally were subjected to hypotonic shock to lyse red blood cells. Total cell numbers were determined by microscopic observation of trypan blue-stained cells using a Neubauer haemocytometer.

Ribosylation assay

Freshly prepared thymocytes were washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in ice-cold sonication buffer [0.5 M sucrose, 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml chymostatin] at a cell concentration of 10^6 /ml and lysed by sonication. ADP-ribosylation was carried out as described previously (Aktories *et al.*, 1992) in a total volume of 200 µl containing 15–20 µg of cell lysate, 0.3 mM GTP, 50 mM NaCl, 1 µCi of [³²P]NAD (NEN DuPont, 800 Ci/mmol) and 40 ng of recombinant C3. The reaction was performed at 37°C for 60 min and stopped by addition of 35 µl of 70% trichloroacetic acid (TCA). Samples were separated by 12% SDS-PAGE and the dried gel subjected to autoradiography.

Flow cytometric analysis

Antibodies (Pharmingen) were conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotin. Biotinylated antibodies were revealed using streptavidin-TRICOLOR (Caltag). Thymocytes or splenocytes were stained for surface expression of CD8 (53-5.8), CD4 (RM4-5), CD25 (IL-2Rαchain, 3C7), CD3ε (145-2C11), αβTCR (β-chain, H57-597), B220 (CD45R, RA3-6B2), CD44 (Pgp-1, IM7), CD2 (LFA-2, RM2-5), HSA (CD24, M1/69), c-Kit (CD117, 2B8), Thy-1.2 (CD90.2, 30-H12), Mac-1 (α-chain, M1/70), Gr-1 (Ly-6G, RB6-8C5) and HY-TCR (T3.70). Cells were stained with saturating concentrations of antibody at 4°C for 30 min at 1×10^6 cells per sample in a 96-well V-bottom shaped microtitre well plate in 100 µl of PBS containing 1% bovine serum albumin. Cells were washed with this buffer in between incubations and prior to analysis on a FACScan (Becton Dickinson). Events were collected and stored ungated in list-mode using CellQuest software. Live cells were gated according to their FSC and SSC profiles and data were analysed using CellQuest software. Data shown are representative of at least five experiments.

Apoptosis assay

Freshly prepared thymocytes were resuspended in PBS at a concentration of 1×10^6 /ml and stained with 200 µl of propidium iodide (50 µg/ml) and 100 µl of Hoechst 33342 (10 µg/ml) for 2 min at room temperature. Events were collected and analysed on a FACS Vantage (Becton Dickinson), using a UV (351–363 nm) laser to excite the Hoechst dye. Propidium iodide fluorescence was measured above 600 nm and Hoechst fluorescence between 390 and 460 nm.

Cell cycle analysis

Cellular DNA content was assayed by standard techniques using propidium iodide staining. Briefly, 1×10^6 thymocytes were stained with FITC-conjugated mAbs against CD4 and CD8 as described and fixed in cold 70% ethanol for 30 min at 4°C. Cells were washed free of ethanol, RNase (1 mg/ml) treated for 15 min at room temperature and resuspended in propidium iodide (50 µg/ml) to stain cellular DNA. Events were collected and analysed on a FACScan (Becton Dickinson) using a doublet

discrimination module, and propidium iodide fluorescence was measured above 600 nm.

In vitro proliferation of isolated CD4⁺ thymocytes

Freshly isolated thymocytes from control and C3 thymi were stained under sterile conditions for CD4 and CD8 expression and subjected to fluorescence-activated cell sorting on a FACS Vantage (Becton Dickinson). CD4⁺ SP cells were collected and seeded in duplicates at 5×10^4 cells/well in 96-well flat bottom microtitre plates (Nunc) in 200 µl of medium (RPMI1640, 10% fetal calf serum, 50 µM 2-mercaptoethanol). Cells were stimulated for 48 h with medium alone or with anti-CD3 mAb (145.2C11, 10 µg/ml in PBS, immobilized on plastic overnight at 4°C) alone or in the presence of IL-2 (20 ng/ml). Thymocytes were labelled with 1 µCi of [³H]thymidine (Amersham), incubated for a further 12–18 h and harvested onto glass fibre filter mates (Pharmacia). [³H]Thymidine incorporation was measured using a microplate scintillation counter (Beckman).

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