Involvement of p21^{ras} distinguishes positive and negative selection in thymocytes

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Small molecular weight GTP binding proteins of the ras family have been implicated in signal transduction from the T cell antigen receptor (TCR). To test the importance of p21^{ras} in the control of thymocyte development, we generated mice expressing a dominant-negative p21^{ras} protein (H-rasN17) in T lineage cells under the control of the *lck* proximal promoter. Proliferation of thymocytes from *lck*-H-rasN17 mice in response to TCR stimulation was nearly completely blocked, confirming the importance of p21^{ras} in mediating TCR-derived signals in mature CD4⁺8⁺ or CD8⁺4⁺ thymocytes. In contrast, some TCR-derived signals proceeded unimpaired in the CD4⁺8⁺ thymocytes of mice expressing dominant-negative p21^{ras}. Analysis of thymocyte development in mice made doubly transgenic for the H-Y-specific TCR and *lck*-H-rasN17 demonstrated that antigen-specific negative selection occurs normally in the presence of p21^{H-rasN17}. Superantigen-induced negative selection *in vivo* also proceeded unhindered in H-rasN17 thymocytes. In contrast, positive selection of thymocytes in the H-Y mice was severely compromised by the presence of p21^{H-rasN17}. These observations demonstrate that positive and negative selection, two conceptually antithetical consequences of TCR stimulation, are biochemically distinguishable.

Key words: ras/signal transduction/thymocyte selection/T cell receptor

Introduction

Antigen recognition by T cells is mediated by heterodimeric receptor structures, typically composed of α and β chains, which recognize peptide antigens bound to class I or class II antigen presentation molecules encoded within the major histocompatibility complex (MHC). The T cell antigen receptor (TCR) α and β chains, whose combined sequences determine the unique antigen specificity of each individual T cell, are themselves encoded by gene segments, positioned discontinuously in germline DNA, that are juxtaposed by site-specific DNA rearrangements during intrathymic T cell development (Kronenberg *et al.*,

1986). Each $\alpha\beta$ heterodimer (and the analogous $\gamma\delta$ heterodimers that are expressed on a specialized minority subpopulation of T cells) associates physically and functionally with a set of non-polymorphic proteins termed the CD3 complex, composed of γ , δ , and ϵ chains, and with a disulfide linked dimer of ζ , or ζ and η , chains (Bluestone *et al.*, 1987). Together, this aggregate of transmembrane molecules both stabilizes the $\alpha\beta$ heterodimer and also participates in delivering TCR-derived signals to the cell interior (Ashwell and Klausner, 1990; Perlmutter *et al.*, 1993). This signaling function depends upon interaction with, and activation of, membrane-associated protein tyrosine kinases (Weiss, 1993).

Signaling from the TCR provokes any one of several different cellular responses depending on the stage of differentiation of the T cell and on its prior antigen recognition history. This heterogeneity of outcomes provoked by stimulation of a single class of receptor is conceptually perplexing and nowhere more so than in the process of antigen-driven thymocyte selection, wherein only those cells bearing receptors capable of recognizing 'foreign' peptides presented by 'self' class I or class II MHC molecules pass to the peripheral lymphoid circulation (Marrack *et al.*, 1993). Selection begins in thymocytes that simultaneously express both the CD4 and the CD8 co-receptor molecules. In these cells, ligand occupancy of the TCR yields two very different outcomes: thymocytes with TCRs that bind peptide and MHC molecules in the thymus 'inappropriately' (often understood as 'with too high an avidity') undergo activation-induced cell death (Kisielow *et al.*, 1988a; Iwabuchi *et al.*, 1992), while those that interact with peptide and MHC molecules correctly are positively selected for survival (von Boehmer, 1992; Hogquist *et al.*, 1993). The existence of these selective events has been extensively documented and recent studies performed under conditions of limiting antigen demonstrate that the extent of ligand interaction with the TCR can by itself determine whether positive or negative selection obtains (Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994; Sebzda *et al.*, 1994). Nevertheless, it remains puzzling that signals transmitted through the same receptor in seemingly identical cells may provoke either death or survival. Indeed, even in mature circulating T cells, TCR stimulation may provoke proliferation, engender a state of unresponsiveness (anergy) to subsequent stimulation (Celis and Saibara, 1992; Kang *et al.*, 1992) or may once again result in cell death (Rocha and von Boehmer, 1991), apparently depending upon the constellation of signals that impinge upon the T cell at the time of interaction with antigen (Harding *et al.*, 1992). Hence, the biochemical signals elicited by ligand occupancy of the TCR must either vary with the stimulating antigen (Racioppi *et al.*, 1993) or must be interpreted flexibly in the context of other receptor-mediated signaling processes.

Fig. 1. The *lck*-H-rasN17 transgene directs p21^{H-rasN17} expression in thymocytes. (A) Diagram of the *lck*-H-rasN17 transgene. The p1017 vector (Chaffin *et al.*, 1990) includes a 3.2 kb fragment of the mouse *lck* gene that contains the proximal promoter to +37 with respect to the transcription start site, juxtaposed with human growth hormone (hGH) genomic sequence (alternating filled and open segments represent exons and introns respectively). The H-rasN17 cDNA was inserted into the first exon of the hGH gene within what is ordinarily 5' untranslated region sequence. (B) Expression of p21^{ras} protein in wild-type and transgenic thymocytes. Shown is an immunoblot performed using 20 μ g of total cellular protein comparing control (non-transgenic) (C), 15465 line (65) and 15728 line (28) thymocyte proteins visualized using a ras-specific antibody. The transgene-derived protein migrates slightly more rapidly than does the endogenous p21^{ras} in the 12% SDS-PAGE system used here.

Insights into TCR signaling circuitry have emerged through analysis of non-receptor protein tyrosine kinases expressed in lymphocytes. In particular, p59^{lyn}, a form of the src family kinase p59^{lyn} that is expressed exclusively in hematopoietic cells, interacts directly with the TCR complex (Cooke and Perlmutter, 1989; Samelson *et al.*, 1990; Gassman *et al.*, 1992), becomes activated following cross-linking of the TCR with anti-CD3 reagents (Yu *et al.*, 1992) and is required for satisfactory TCR-induced mitogenesis in mature CD4⁺8⁻ or CD4⁻8⁺ thymocytes (Cooke *et al.*, 1991; Appleby *et al.*, 1992; Stein *et al.*, 1992). The closely related kinase p56^{lck} also appears to participate in regulating antigen-induced T cell activation, in part through its interaction with CD4 and CD8 co-receptor molecules (Rudd *et al.*, 1988; Veillette *et al.*, 1988, 1989; Shaw *et al.*, 1989; Turner *et al.*, 1990; Xu and Littman, 1993). This kinase may also interact with the TCR complex itself (Straus and Weiss, 1992; Anderson *et al.*, 1993; Salcedo *et al.*, 1993). Finally, a third protein tyrosine kinase, ZAP-70, interacts with the ζ homodimer and is required for satisfactory T cell development and signaling (Arpaia *et al.*, 1994; Elder *et al.*, 1994).

To address mechanisms that might permit coupling of the TCR to multiple distinct signaling pathways, we sought to selectively perturb the function of molecules which respond to the activation of protein tyrosine kinases. Among the large set of molecules believed to relay protein tyrosine kinase-derived signals, p21^{ras} is an especially appropriate target for investigation, since it is known to participate in signaling from a wide variety of protein tyrosine kinases, including those of the src family (Sternberg and Horvitz, 1989; Simon *et al.*, 1991; Qureshi *et al.*, 1992). Studies of lymphocyte activation have demonstrated accumulation of p21^{ras} in its active, GTP-bound form following TCR cross-linking (Downward *et al.*, 1990), an effect that may reflect tyrosine kinase-stimulated increases in ras-specific GTP exchange activity (Izquierdo *et al.*, 1992), perhaps mediated by the vav protein (Gulbins *et al.*, 1993). Moreover, several reports demonstrate that expression of a dominant-negative form of p21^{ras} in transformed T cell lines can block activation of the interleukin-2 (IL-2) gene following TCR cross-linking (Rayter *et al.*, 1992; Baldari *et al.*, 1993; Woodrow *et al.*, 1993). Together these studies suggested that compromising p21^{ras} function in otherwise normal thymocytes would block protein tyrosine kinase signaling pathways known to function in these cells.

Here we report that expression of a transgene encoding a dominant-negative p21^{ras} protein in T lineage cells

substantially blocks TCR-mediated positive selection without compromising the negative selection of thymocytes that can be induced by stimulating the same TCR with a different ligand. Hence, differences in outcome following ligand occupancy of the TCR appear to reflect flexibility in the coupling of downstream signal transducing molecules, notably p21^{ras}.

Results

To inhibit *ras* activation in thymocytes, transgenic mice were generated in which a dominant-negative mutant of p21^{ras}, H-ras N17 (with asparagine substituted for serine at position 17), was expressed at high levels under the control of the *lck* proximal promoter using the p1017 expression vector (Chaffin *et al.*, 1990). The activity of the *lck* proximal promoter is such that detectable transgene expression is restricted to thymocytes; the promoter is most active in immature cells and is almost silent in mature peripheral T cells (Wildin *et al.*, 1991). Figure 1A provides a schematic diagram of the *lck*-H-rasN17 expression construct, which includes a 3.2 kb fragment of the *lck* proximal promoter, a 0.7 kb H-rasN17 cDNA insert and 2.1 kb of genomic sequence derived from the human growth hormone gene, which provides a polyadenylation signal and an exon/intron structure that can in many cases improve transgene expression (Brinster *et al.*, 1988). A 6 kb *NotI* fragment containing the expression construct was injected into (C57BL/6J $\times$ DBA/2) F2 embryos using standard techniques and five founder mice were generated. Of these, two (15465 and 15728) whose progeny expressed the transgene at high levels were backcrossed to C57BL/6J mice to generate lines.

In normal animals, thymocytes contain no detectable protein encoded by the endogenous mouse H-ras gene and little p21^{ras} protein encoded by either of the other two *ras* isoforms, K-ras and N-ras (Figure 1B and data not shown) (Leon *et al.*, 1987). In contrast, thymocytes from *lck*-H-rasN17 mice contain very high levels of immunoreactive p21^{ras} protein (Figure 1B). Expression of this protein had no appreciable effect on thymocyte number or on the accumulation of CD4⁺8⁺ cells, which ordinarily represent the bulk of thymocytes and in which TCR expression first appears (see below). We therefore examined the effects of p21^{H-rasN17} on thymocyte signaling.

Dominant-negative H-ras blocks TCR-mediated T cell activation

Previous studies in transformed human T cell lines have demonstrated that expression of H-rasN17 substantially

Fig. 2. Decreased proliferative responses in thymocytes from *lck-H-rasN17* mice. Thymocytes were stimulated with concanavalin A (CA) with or without PMA, 145.2C11 anti-CD3e ascites (CD3) with or without PMA, or ionomycin with PMA and proliferation analyzed by measuring incorporation of [3 H]thymidine. Counts per min (c.p.m.) detected by scintillation counting were normalized for the percentage of CD4 $^{+}$ 8 $^{-}$ cells in the thymus as determined by flow cytometry. Each assay was performed in triplicate. This experiment is representative of 10 such independent experiments.

disrupts signaling from the TCR. Under these circumstances, transcriptional activation of the IL-2 gene, and in particular the activity of nuclear factor of activated T cell (NFAT)-specific *cis*-acting transcriptional regulatory sequences, is blocked almost completely (Rayter *et al.*, 1992; Baldari *et al.*, 1993; Woodrow *et al.*, 1993). Similarly, *lck-H-rasN17* thymocytes failed to proliferate in response to stimulation using anti-CD3 antibodies or the mitogen concanavalin A, either alone or in the presence of phorbol 12-myristate, 13 acetate (PMA), provided as a co-stimulator (Figure 2). Interestingly, although the combination of a calcium ionophore and phorbol esters (a regimen that bypasses the TCR) failed to stimulate EL4 thymoma cells transiently expressing H-rasN17 (Rayter *et al.*, 1992), these agents satisfactorily induced proliferation in *lck-H-rasN17* thymocytes (Figure 2). Thus, in otherwise normal thymocytes high-level expression of p21 $^{H-rasN17}$ specifically interdicts signaling from the TCR.

The activation defect in *lck-H-rasN17* thymocytes is reminiscent of that observed in thymocytes from mice in which the function of p59 fyn has been compromised (Cooke *et al.*, 1991; Appleby *et al.*, 1992). For example, thymocytes from mice made homozygous for a *fyn* null allele exhibit defective proliferative responses to TCR stimulation while responding normally to combinations of ionomycin and phorbol esters (Appleby *et al.*, 1992). We therefore examined other TCR-induced responses in thymocytes from *lck-H-rasN17* mice. Figure 3 demonstrates that TCR-induced accumulation of phosphotyrosine-bearing proteins remains intact in thymocytes expressing dominant-negative *ras*. This suggests that protein tyrosine kinase activation proceeds normally following TCR stimulation in *lck-H-rasN17* thymocytes. Similarly, in mature CD4 $^{+}$ 8 $^{-}$ cells, as in all thymocyte subsets, calcium mobilization, which in part reflects the activation of phospholipase C γ 1 by tyrosine phosphorylation following TCR cross-linking (Premack and Gardner,

Fig. 3. TCR-induced phosphotyrosine accumulation in thymocytes from *lck-H-rasN17* mice. Total thymocytes were stimulated with anti-CD3e antibodies and PMA and protein lysates (representing $\sim 2 \times 10^6$ cell equivalents) derived from cells following stimulation for the indicated times were removed and fractionated by SDS-PAGE using an 11% gel system. Following transfer, the phosphotyrosine-containing proteins were visualized as described in Materials and methods. The response of normal littermate control cells is compared with that of transgenic cells.

1992; Archuleta *et al.*, 1993), also occurs normally (Figure 4 and data not shown). Thus, the earliest biochemical signals provoked by TCR stimulation are not detectably affected by p21 $^{H-rasN17}$.

However, defective recruitment of more distal signaling processes can be easily demonstrated. Thus, induction of MAP kinase activity, a normal concomitant of TCR stimulation (Izquierdo *et al.*, 1993), does not occur in the presence of p21 $^{H-rasN17}$ (Figure 5) and activation of the S6 kinase, a presumed target of MAP kinase regulation (Ahn *et al.*, 1992), is similarly compromised (data not shown). These data permit the positioning of p21 ras within the circuitry underlying T cell activation (see below).

Negative selection is intact in the presence of p21 $^{H-rasN17}$

Although the proliferative response of thymocytes to anti-CD3 stimulation *in vitro* is almost completely blocked by the presence of p21 $^{H-rasN17}$, thymocyte development, assessed simply by examining the numbers of cells bearing CD4 and CD8 co-receptors, was largely unaffected. Figure 6 demonstrates that the representation of immature CD4 $^{+}$ 8 $^{+}$ cells and of their CD4 $^{+}$ 8 $^{-}$ progenitors was unperturbed by the presence of the *lck-H-rasN17* transgene. In contrast, the single-positive (CD4 $^{+}$ 8 $^{-}$ and CD4 $^{-}$ 8 $^{+}$) CD3 hi progeny of CD4 $^{+}$ 8 $^{+}$ cells, products of positive selection, were consistently reduced in number by 40–60% (Figure 7A and B). This effect appeared to depend upon the dose of p21 $^{H-rasN17}$, in that thymi from animals expressing the higher transgene level were typically more severely affected (CD4 $^{+}$ 8 $^{-}$ cells were represented at 38 ± 6.9 or $63 \pm 11.4\%$ of normal values for 10 animals of the 65 and 28 lines respectively).

In principle, the reduced representation of single-positive cells in *lck-H-rasN17* thymocytes might result from one of several different mechanisms, e.g. ineffective positive selection by appropriate TCR ligands, over vigorous TCR-induced negative selection or a non-

Fig. 4. Normal calcium mobilization in *lck*-H-*ras*N17 thymocytes. Intracellular free calcium levels were measured by flow cytometric analysis of Indo-1-labeled thymocytes. The ratio of violet:blue fluorescence of Indo-1, which is proportional to free calcium concentration, is plotted versus time following stimulation with anti-CD3 ϵ antibodies for thymocytes from a *lck*-H-*ras*N17 animal or a normal littermate control. Shown are data obtained by gating electronically on CD4⁺8⁻ cells.

selection-associated developmental block. To characterize the reduction in the representation of mature thymocytes associated with H-*ras*N17 expression in more detail, we studied the deletion by negative selection of cells bearing V β 6⁺ TCR β chains, a deletion that occurs in mice which simultaneously express a mouse mammary tumor virus (Mtv-7)-encoded superantigen and the I-E Class II MHC molecule (Gollob and Palmer, 1991). This was achieved by backcrossing *lck*-H-*ras*N17 mice (nominally I-E⁻ and Mtv-7⁻) to DBA/2 mice (I-E⁺, Mtv-7⁺). Previous studies demonstrate that virtually all V β 6-bearing thymocytes are deleted in I-E⁺Mtv-7⁺ mouse strains as CD4⁺8⁺ cells mature (Hengartner *et al.*, 1988). This deletion process results from the binding of the superantigen/class II protein complex to the V β 6 TCR (Marrack *et al.*, 1993) and is felt to mimic antigen-specific negative selection (Jenkinson *et al.*, 1989). As shown in Figure 7, deletion of V β 6-bearing cells occurs as efficiently in I-E⁺Mtv-7⁺ *lck*-H-*ras*N17 mice as in littermate control mice lacking the *lck*-H-*ras*N17 transgene. Hence, in this model system, TCR-derived signals mediating negative selection are not compromised by high-level expression of dominant-negative p21^{H-rasN17}.

To determine whether peptide-mediated negative selection occurred normally in the presence of p21^{H-rasN17}, we crossed the *lck*-H-*ras*N17 mice with mice bearing a transgene encoding TCR α and β chains that together specify reactivity with the male (H-Y) antigen presented by H-2 D^b class I molecules. Previous studies have shown that thymocytes in H-2^b, anti-H-Y $\alpha\beta$ male mice are deleted early in the maturation process, reflecting contact between H-Y-specific TCRs and the H-Y peptide bound by D^b MHC molecules (Kisielow *et al.*, 1988a; Swat *et al.*, 1991). Conversely, in anti-H-Y $\alpha\beta$ transgenic female mice, a positively selecting combination of peptide and MHC results in more efficient maturation of CD4⁺8⁺ T cells (Kisielow *et al.*, 1988b; Teh *et al.*, 1988). Male and female (*lck*-H-*ras*N17 $\times$ anti-H-Y $\alpha\beta$) progeny between the ages of 3 and 6 weeks were therefore analyzed by three-color

Fig. 5. Defective activation of MAP kinases in CD4⁺8⁻ thymocytes from *lck*-H-*ras*N17 mice. Purified CD4⁺8⁻ cells were stimulated with anti-CD3 ϵ antibodies for 10 min, then sonicated in the presence of protease and phosphatase inhibitors. Shown is the mean relative increase in phosphorylation of myelin basic protein with respect to unstimulated control cells following subtraction of background counts for three independent experiments performed using *lck*-H-*ras*N17 thymocytes or control cells. The error bars denote one standard deviation from the mean.

flow cytometry for the effect of the dominant-negative *ras* transgene on thymocyte selection.

Figure 8 presents CD4 versus CD8 profiles of thymocytes expressing high levels of the transgenic α chain maturing in these male progeny. As expected, a virtually complete absence of CD4⁺8⁺ cells was observed in anti-H-Y $\alpha\beta$ transgenic mice, reflecting the elimination of self-reactive thymocytes. Remarkably, this negative selection process occurred equally well in mice that simultaneously expressed the dominant-negative *ras* transgene (Figure 8). Hence, in two independent assay systems, high-level expression of p21^{H-rasN17} does not influence the TCR-derived negative selection signal. The inability of p21^{H-rasN17} to perturb negative selection does not result from inefficient expression of the *lck*-H-*ras*N17 transgene in immature cells. Indeed, the *lck* proximal promoter is especially active in these immature populations (Wildin *et al.*, 1991) and levels of p21^{H-rasN17} were higher in the CD4⁺8⁻ and CD4⁺8⁺ cells of *lck*-*ras*N17 mice than in the non-responsive single-positive cells that ordinarily proliferate following TCR stimulation (data not shown). It therefore appears that transgene-directed expression of p21^{H-rasN17} permits discrimination between various TCR-induced signaling outcomes; proliferative responses of mature thymocytes are blocked, while antigen-specific negative selection remains intact.

Attenuated positive selection in *lck*-H-*ras*N17 mice

To assess further the effects of dominant-negative *ras* expression on TCR signaling, positive selection of thymocytes bearing H-Y-specific TCRs was also examined. In female H-Y $\alpha\beta$ TCR mice, the representation of CD4⁺8⁺ thymocytes increases substantially, reflecting the high likelihood that thymocytes bearing this TCR will react

Fig. 6. Reduced representation of mature $CD3^{hi}$ thymocytes in *lck-H-rasN17* mice. Shown are fluorescence histograms for thymocytes from a 15728 animal, a 15465 animal and a sex-matched littermate control. (Top) Thymocytes were stained with 500AA2 monoclonal antibody to reveal CD3e expression. (Bottom) Dual-parameter histograms showing CD4 and CD8 expression.

with peptides presented by the D^b class I molecule (Kisielow *et al.*, 1988b; Teh *et al.*, 1988; Huesmann *et al.*, 1991). Positive selection in this system cannot be analyzed simply by studying the representation of cells bearing CD4 and CD8, however, because of superimposed endogenous TCR α gene rearrangement (Borgulya *et al.*, 1992). This difficulty can be alleviated by gating electronically on those cells expressing high levels of the transgene-encoded receptor, thereby permitting analysis of thymocytes actually undergoing positive selection. Figure 9 (top panel) shows that in mice simultaneously expressing both the H-Y-specific TCR and the *lck-H-rasN17* transgene the generation of $CD4^+8^+$ cells is reduced by $>85\%$ as compared with littermate H-Y TCR controls, however, inasmuch as the TCR α chains in such cells are potentially heterogeneous, it is difficult to assess the magnitude of this reduction in antigen-specific selection.

To establish more precisely the effects of the *lck-H-rasN17* transgene on positive selection, additional flow cytometric analyses were performed. Swat *et al.* (1992) have identified a subset of $CD4^+8^+$ thymocytes, small cells expressing high levels of the TCR, which appear to represent a post-selection population based on their ability to differentiate into $CD8^+4^-$ cells *in vitro*. Using this

Fig. 7. Superantigen-mediated deletion of $V\beta6$ -bearing thymocytes. Shown is a single parameter histogram plotting cell number (Y axis) versus $V\beta6$ staining in $CD3^{hi}$ cells (identified using simultaneous anti-CD3e staining). The three superimposed profiles reflect results obtained with normal C57BL/6/J female thymocytes as compared with those derived from littermate F1 progeny of a *lck-H-rasN17* $\times$ DBA/2/J cross. The presence of the transgene was assessed by Southern blotting of tail DNA. The lowest channels on the $V\beta6$ axis were gated out to remove the $V\beta6^-$ cells, which would otherwise shift the axis.

Fig. 8. H-Y $\alpha\beta$ -directed deletion of thymocytes in *lck*-H-*ras*N17 mice. Thymocytes from male F1 progeny of H-Y $\alpha\beta$ homozygous $\times$ *lck*-H-*ras*N17 heterozygous crosses which either have or have not acquired the *lck*-H-*ras*N17 transgene are compared. Thymocytes were prepared as previously described and stained with anti-CD4-PE, anti-CD8-FITC and T3.70 (anti-H-Y α) conjugated to biotin. The biotinylated antibody was detected using streptavidin-Tricolor. Ungated list mode events (100 000) were collected and analyzed as previously described. Shown are the CD4 and CD8 staining profiles of T3.70⁺ cells. This analysis represents one of nine such pairs of mice analyzed.

information, the efficacy of positive selection of H-Y-specific thymocytes can be more carefully assessed. Examination of the thymocytes that express high levels of the transgene-encoded α chain, achieved using the T3.70 antibody described by Teh *et al.* (1990), reveals that in control anti-H-Y $\alpha\beta$ mice the small TCR^{hi} cells include CD8⁺ thymocytes distributed over a broad range of CD4 expression levels (Figure 9, middle panel). A small proportion (typically <10%) of these cells are CD4⁻8⁻ and may represent products of a distinct maturation pathway (von Boehmer *et al.*, 1991). Remarkably, while the representation of these CD4⁻8⁻ TCR^{hi} cells is not compromised by the presence of the *lck*-H-*ras*N17 transgene, the generation of positively selected CD4⁺8⁺ cells is almost completely blocked (Figure 9, middle panel). Thus, *lck*-H-*ras*N17 thymocytes contain reduced numbers of single-positive cells and this reduction can be localized, at least in the case of cells expressing the H-Y-specific TCR, to the stage at which CD4⁺8⁺ cells

receive a positively-selecting signal following TCR stimulation.

Distinctions between p59^{fyn}-mediated and p21^{H-ras}N17-inhibited TCR signals

Because high-level expression of the *lck*-H-*ras*N17 transgene leads to a block in the proliferation of mature thymocytes responding to TCR-derived signals (Figure 2) and since a similar block is observed in animals expressing a dominant-negative form of p59^{fyn} (Cooke *et al.*, 1991) or lacking p59^{fyn} altogether (Appleby *et al.*, 1992; Stein *et al.*, 1992), it seemed plausible that the inhibition of positive selection achieved in *lck*-H-*ras*N17 mice might also occur in thymocytes from p59^{fyn}-deficient mice. Unlike *lck*-H-*ras*N17 mice, however, single-positive thymocytes are represented in normal numbers in mice bearing a targeted disruption of the *fyn* gene (Appleby *et al.*, 1992). To compare positive selection in *fyn*-deficient mice and in mice bearing the dominant-negative *lck*-H-

Fig. 9. Defective positive selection of H-Y $\alpha\beta$ ⁺ thymocytes in *lck*-H-*rasN17* mice. Thymocytes from female progeny of F1 H-Y $\alpha\beta$ homozygous $\times$ *lck*-H-*rasN17* heterozygous crosses were analyzed for CD4, CD8 and T3.70 expression. List mode events (100 000) were collected and analyzed as previously described. (A) CD4/CD8 dual-parameter histograms ungated with respect to TCR expression. The percentage of events in each quadrant is shown. (B) CD4/CD8 dual-parameter histograms for cell populations defined as anti-H-Y α ^{hi} with low forward scatter are compared. The numbers of events are displayed in each quadrant. (C) The analysis performed in (B) was repeated with H-Y $\alpha\beta$ animals that were bred to be either *fyn*T⁺/*fyn*T⁺ or *fyn*T^{null}/*fyn*T^{null}.

rasN17 transgene more directly, we mated *fyn*T^{null} mice (Appleby *et al.*, 1992) with anti-H-Y $\alpha\beta$ TCR transgenic mice and backcrossed these to obtain animals bearing the H-Y-specific TCR transgene but lacking p59^{*fyn*T} expression. Figure 9 (bottom panel) shows that positive selection of thymocytes expressing high levels of the H-Y-specific TCR transgene occurs normally even in the absence of p59^{*fyn*T} expression. This observation is consistent with our previous studies demonstrating that p59^{*fyn*T} expression remains low until maturation to the single-positive stage occurs (Cooke *et al.*, 1991). Hence, dominant-negative

p21^{*ras*} antagonizes some signaling pathways that do not involve p59^{*fyn*}.

Discussion

Involvement of p21^{*ras*} in protein tyrosine kinase signaling

Investigation of p21^{*ras*} function has been greatly aided by the discovery of mutants that act in a dominant-negative fashion, presumably by binding competitively to nucleotide exchange factors and hence preventing accumulation

of GTP-bound wild-type p21^{ras} (Feig and Cooper, 1988; Schweighoffer *et al.*, 1993). Previous studies revealed that high-level expression of one such mutant *ras* gene, encoding H-rasN17, blocked the delivery of TCR-derived signals leading to IL-2 expression in two different human leukemic T cell lines (Rayter *et al.*, 1992; Baldari *et al.*, 1993). These observations, coupled with the demonstration that GTP-bound p21^{ras} accumulates following TCR or lymphokine receptor stimulation (Downward *et al.*, 1990; Graves *et al.*, 1992; Satoh *et al.*, 1992) in a manner that can be blocked by protein tyrosine kinase inhibitors (Izquierdo *et al.*, 1992), strongly suggested that p21^{ras} might serve to relay signals derived from protein tyrosine kinases that interact with the TCR.

Our observations add further support to this hypothesis. In particular, we found that thymocytes expressing high levels of p21^{H-rasN17} were incapable of mounting a proliferative response following TCR stimulation (Figure 2). A similar proliferative defect is associated with compromised activation of a TCR-associated protein tyrosine kinase, p59^{lyn} (Appleby *et al.*, 1992; Stein *et al.*, 1992). Since both protein tyrosine phosphorylation and calcium mobilization occur to roughly the same extent and with the same kinetics in thymocytes from *lck-H-rasN17* mice and normal mice, it seems plausible that p21^{ras} either transduces the p59^{lyn}-mediated signal or participates in a crucial but parallel signaling pathway in mature, single-positive thymocytes.

Postulating that p21^{ras} serves to relay p59^{lyn} (or other kinase)-derived signals renders TCR signaling harmonious with a now widely accepted paradigm for growth factor receptor-induced proliferation, in which a protein tyrosine kinase stimulates *ras* nucleotide exchange, leading to the recruitment of the *raf*-encoded kinase to the plasma membrane, which, in turn, activates the map kinase kinase (MEK), the MAP kinases (ERKs) and the S6 kinases, thereby promoting entry into the cell cycle (Ahn *et al.*, 1992; Egan *et al.*, 1993; Lane *et al.*, 1993; Stokoe *et al.*, 1994). In particular, we observed that the expression of dominant-negative *ras* in otherwise normal thymocytes blocks MAP and S6 kinase activation, suggesting that these kinases participate in delivering the proliferative signal that devolves from TCR stimulation (Figure 5 and data not shown). This is consistent with results recently reported by Izquierdo *et al.* (1993, 1994). Previous studies have demonstrated that p21^{H-rasN17} can antagonize a broad range of protein tyrosine kinase-stimulated signaling events, including transformation induced by p60^{v-src} (Feig and Cooper, 1988) as well as PDGF- and EGF-induced mitogenesis (Cai *et al.*, 1990; Wood *et al.*, 1992) and vulval cell differentiation mediated by a receptor-type protein tyrosine kinase (*let-23*) in *Caenorhabditis elegans* (Sternberg and Horvitz, 1991; Satoh *et al.*, 1992). However, our analysis reveals that not all TCR-derived signaling processes are compromised by the presence of dominant-negative p21^{ras}.

Intrathymic TCR signaling may in some instances be *ras* independent

The preservation of both increased phosphotyrosine accumulation and calcium mobilization in thymocytes expressing p21^{H-rasN17} is unsurprising, since these responses are positioned (in most experimental systems)

proximal to the point at which p21^{ras} activation occurs. However, superantigen-mediated and peptide-specific negative selection proceed normally in *lck-H-rasN17* mice. Hence TCR-derived signals leading to negative selection may not employ a *ras*-mediated pathway. Similarly, the maturation of CD4⁺8⁺ cells from CD4⁺8⁻ precursors also appears to be insensitive to the effects of p21^{H-rasN17}, despite the fact that this developmental event is dependent upon the activation of p56^{lck} (Molina *et al.*, 1992; Anderson *et al.*, 1993; Mombaerts *et al.*, 1994).

Although the signals underlying positive and negative selection appear qualitatively distinct, quantitative arguments, which posit that successful negative selection and p56^{lck}-mediated maturation require little residual p21^{ras} activity as compared with positive selection, remain tenable (Hogquist *et al.*, 1994; Sebzda *et al.*, 1994). We also note that thymocytes express primarily N-*ras* and K-*ras*. It is possible that p21^{H-rasN17} fails to inhibit some signaling processes mediated by the other *ras* isoforms. These considerations notwithstanding, clonal deletion initiated by TCR stimulation can clearly occur in the presence of levels of p21^{H-rasN17} shown to completely inhibit other TCR-derived responses in the very same cells.

Positive selection in *lck-H-rasN17* mice

Recent data support the hypothesis that variations in TCR avidities for peptide/MHC ligands determine the outcome of thymocyte selection (Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994; Sebzda *et al.*, 1994). This type of model leaves open the question of how the 'strength' of signals from the thymocyte surface can be assessed intracellularly. Our data indicate that p21^{ras} activation assists in relaying signals that direct the selection process. Alternative hypotheses, which argue that p21^{H-rasN17} affects development itself, rather than TCR-mediated selection, are much less attractive, since the most carefully characterized precursors of positively-selected CD4⁺8⁺ cells are also targets for negative selection (Guidos *et al.*, 1990; van Meerwijk and Germain, 1993). Thus, the data presented here are compatible with the view that a 'weak' positively selecting stimulus, generated by the H-Y-specific TCR, is effectively blocked by p21^{H-rasN17}, while stronger signaling events, which direct negative selection, are not substantially compromised. Those single-positive thymocytes that do emerge in *lck-H-rasN17* mice may have experienced a 'signal conversion' event, wherein partial inhibition of negative selection permits positive selection to proceed instead, or, alternatively, may express TCRs that deliver signals just strong enough to overcome the effects of p21^{H-rasN17}.

Regardless of the precise mechanism involved, TCR-derived signals responsible for thymocyte selection, and for proliferative responses in more mature cells, can clearly be distinguished biochemically by virtue of their differing sensitivities to p21^{H-rasN17}. Very similar results have also been obtained in thymocytes expressing high levels of catalytically inactive MEK1, a MAP kinase kinase (J.Alberola-Ila, K.Forbush and R.M.Perlmutter, unpublished data), supporting the importance of the *ras* pathway in thymocyte signaling. While cyclosporin A, by inhibiting calcineurin, can inhibit negative selection, positive selection and T cell activation (Gao *et al.*, 1988; Schreiber and Crabtree, 1992; Bucy *et al.*, 1993),

p21^{H-rasN17} separates TCR-induced responses into distinct categories. Our results encourage the hypothesis that these easily distinguishable outcomes of T cell signaling in thymocytes, and perhaps the differential stimulation of apoptosis, anergy or activation in mature cells, may result from differences in the extent to which p21^{ras} becomes activated.

Materials and methods

Transgenic mouse generation

The H-rasN17 transgene was produced by ligation of the 0.7 kb H-rasN17 cDNA (containing 27 bp of 5' untranslated region, the entire coding region and 170 bp of 3' untranslated region sequence) into the BamHI cloning site of the p1017 vector (Chaffin *et al.*, 1990). Purification and injection at 5 ng/ μ l of the NotI fragment containing the H-rasN17 transgene into (C57BL6/J $\times$ DBA/2J) F2 mouse zygote pronuclei was performed as previously described (Garvin *et al.*, 1990). Transgenic founders were detected by hybridization of genomic DNA with an hGH probe and stable lines of mice were generated by backcrossing founders with C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME).

Thymocyte proliferation

Live thymocytes (4×10^5) were plated in 200 μ l of media [RPMI 1640, 10% fetal calf serum (Hyclone), 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin]. Cells were stimulated with mitogens at the following concentrations: 1 ng/ml PMA (Sigma Chemical Co., St Louis, MO), 0.3 μ g/ml concanavalin A (Vector Laboratories), 145.2C11 ascites (anti-CD3e) at a 1:1000 dilution, 500 ng/ml ionomycin (Calbiochem) or some combination of the above. After culture with mitogen for 48 h, cells were labeled with 1 μ Ci [³H]thymidine (Amersham) for 12–18 h. Harvesting and quantitation of [³H]thymidine incorporation was accomplished as previously described (Appleby *et al.*, 1992).

Calcium analysis

Calcium mobilization within thymocytes was measured as previously described (Cooke *et al.*, 1991). Briefly, cells loaded with Indo-1 (Molecular Probes, Junction City, OR) were stained with azide-free anti-CD4 (phycoerythrin-conjugated GK1.5; Becton-Dickinson, Mountain View, CA) and anti-CD8 (fluorescein isothiocyanate-conjugated 53-6.71; Becton-Dickinson) monoclonal antibodies. These cells were activated by treatment with a 1:200 dilution (previously determined to be a sub-maximal dose) of 145.2C11 ascites. Four-color fluorescence data were collected using a FACSTAR flow cytometer (Becton-Dickinson) for 10 min and analyzed using MTIME software (Rabinovitch *et al.*, 1986).

Immunoblot analyses

To determine levels of phosphotyrosine accumulation, stimulation of thymocytes, preparation of lysates and immunoblotting were carried out as described in Cooke *et al.* (1991), with the exception that the cell lysis buffer also contained 20 μ g/ml aprotinin (Sigma), 20 μ g/ml leupeptin (Boehringer-Mannheim) and 10 μ g/ml soybean trypsin-chymotrypsin inhibitor (Sigma). Following SDS-PAGE, proteins were transferred onto Hybond C-Extra membrane and probed with an anti-phosphotyrosine antibody (Upstate Biotechnology Inc., New York, NY). The blot was developed with the addition of horseradish peroxidase-conjugated sheep anti-mouse secondary antibody and visualized using the Enhanced Chemiluminescence (ECL) Detection system (Amersham). Levels of p21^{ras} and p21^{rasN17} expression in thymocytes were determined via the same method using a pan-ras-specific antibody (ras 10, Ab-3; Oncogene Sciences, Cambridge, MA), developed with the same sheep anti-mouse antibody.

MAP kinase (ERK) activity

CD4⁺8⁺ thymocytes were purified by two rounds of negative panning with a CD8-specific mAb (3-155). The resulting cells (50–70% CD3^{hi}4⁺8⁺) were resuspended in Hank's balanced salt solution (HBSS) and stimulated with a 1/200 dilution of the 145.2C11 (anti-CD3e) ascites preparation. After 10 min, stimulation was stopped with ice-cold HBSS and cells were washed and resuspended in homogenization buffer (50 μ M α -glycerophosphate, pH 7.3, 1.5 mM EGTA, 0.1 mM Na₃VO₄, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM benzamide). Cells were disrupted by sonication followed by centrifuga-

tion at 12 000 g for 10 min at 4°C. The supernatant was applied to a DE52 column and MAPK was eluted with homogenization buffer made 200 mM in NaCl. Fractions were mixed with either 1 mg/ml myelin basic protein (Sigma) or H₂O and the phosphorylation reaction was started by adding assay buffer containing 75 μ M α -glycerophosphate, pH 7.3, 3.75 mM EGTA, 0.1 mM Na₃VO₄, 1.5 mM dithiothreitol, 30 μ M calmidazolium, 6 μ M PKI peptide (a peptide inhibitor of cAMP-dependent kinase), 30 mM MgCl₂ and 0.3 μ M [γ -³²P]ATP. Reactions were incubated for 20 min at 30°C and terminated by spotting 20 μ l of the assay mixture on P81 phosphocellulose filter paper (Whatman), which was washed several times with 175 mM phosphoric acid. Levels of phosphate incorporation in the absence of substrate, determined by scintillation counting, were subtracted from values obtained in the presence of substrate to correct for non-specific phosphorylation.

Flow cytometric analysis

Thymocytes were stained with saturating concentrations of antibody at 4°C for 45 min–1 h at $1\text{--}2 \times 10^6$ cells/well in a 96-well plate in 40 μ l of staining reagent. Cells were stained for surface expression of the following molecules: CD4 (as above), CD8 (as above), CD3e [biotinylated 500AA2 (Havran *et al.*, 1987)] or FITC-conjugated 145.2C11 (Leon *et al.*, 1987)], H-Y specific TCR α chain (biotinylated T3.70; Teh *et al.*, 1988) or V β 6 (biotinylated R44-7; Kanagawa *et al.*, 1989). Biotinylated antibodies were visualized using phycoerythrin- or trichrome-labeled streptavidin (Caltag). Analyses were performed using a FACSCAN flow cytometer (Becton-Dickinson) running Consort 30 or Lysis II software; events were collected and stored without gating in list mode. Subsequent data reduction used these data packages as well as Reproman software (True Facts Software, Seattle, WA).

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