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The Mitogen-Induced Increase in T Cell Size Involves PKC and NFAT Activation of Rel/NF-κB-Dependent c-*myc* Expression

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Summary

Cell growth during the G1 stage of the cell cycle is partly controlled by inducing c-*myc* expression, which in B cells is regulated by the NF-κB1 and c-Rel transcription factors. Here, we show that c-*myc*-dependent growth during T cell activation requires c-Rel and RelA and that blocking this growth by inhibiting protein kinase C theta (PKCθ) coincides with a failure to upregulate c-*myc* due to impaired RelA nuclear import and inhibition of NFAT-dependent c-*rel* transcription. These results demonstrate that different Rel/NF-κB dimers regulate the mitogenic growth of mature T and B cells, with a signaling pathway incorporating PKCθ and NFAT controlling c-Rel/RelA-induced c-*myc* expression in activated T cells.

Introduction

TCR and costimulatory receptor engagement initiate a signaling cascade in T cells that induces changes in gene expression, culminating in these cells becoming immune effectors. During T cell activation, entry of quiescent lymphocytes into the cell cycle is accompanied by an increase in biomass and size. This event, termed blast formation or cell growth, is intimately linked with increased RNA and protein expression that occurs prior to DNA synthesis (Conlon and Raff, 1999). Cell growth and division are tightly coupled, with DNA synthesis and division generally dependent on growth. Growth is thought to proceed throughout the G1 phase of the cell cycle until threshold levels of certain cellular components that function as indicators of cell size trigger entry into S phase (Conlon and Raff, 1999).

Of the intracellular signaling networks implicated in T cell activation, many converge upon the Rel/NF-κB and NFAT pathways (Kane et al., 2002; Masuda et al., 1998). The transcriptional mediators of the Rel/NF-κB pathway comprise dimers of related proteins (c-Rel, RelA(p65), RelB, p50NF-κB1, and p52NF-κB2) that bind consensus κB elements within the regulatory regions of target genes (Baldwin, 1996). In most cells, the main pool of Rel/NF-κB is sequestered to the cytoplasm by inhibitory proteins (IκBs). Diverse stimuli, including mitogens, induce Rel/NF-κB nuclear import by activating an IκB kinase (IKK) complex that phosphorylates IκB proteins (Karin, 1999), targeting them for ubiquitin-dependent proteosome-mediated degradation (Whiteside and Israel, 1997). Members of the NFAT family of transcription factors (NFATC1, NFATC2, NFATC3, NFATC4, NFATC5) also pre-exist in the cytoplasm and are translocated to the nucleus in response to calcium-dependent signals that lead to NFAT dephosphorylation by calcineurin (Rao et al., 1997). Nuclear NFAT proteins bind regulatory elements within target genes in a cooperative fashion with other transcription factors, most notably AP-1 proteins (Rao et al., 1997).

During T cell activation, different Rel/NF-κB dimers enter the nucleus in a temporal fashion. In resting cells, only NF-κB1 homodimers are detected in the nucleus (Bryan et al., 1994). Within 30 min of stimulation, NF-κB1/RelA (p50/p65) heterodimers are translocated to the nucleus from the cytoplasm, whereas significant nuclear levels of c-Rel are only detected ~90 min post-activation (Molitor et al., 1990; Venkataraman et al., 1996). Although some c-Rel resides in the cytoplasm of unstimulated T cells, most of this c-Rel pool is not rapidly mobilized in response to mitogenic signals (Venkataraman et al., 1995). Instead, nuclear c-Rel expression appears to follow the transcriptional induction of c-*rel* (Grumont and Gerondakis, 1990), a finding consistent with its nuclear localization during T cell activation being dependent on new c-Rel synthesis (Venkataraman et al., 1995, 1996). NFATC1 and NFATC2 are the major NFAT isoforms expressed in peripheral T cells (Rao et al., 1997). There is no evidence for the temporal nuclear import of different NFAT proteins during T cell activation.

Gene targeting has revealed the essential roles served by individual Rel/NF-κB and NFAT proteins during T cell activation (Gerondakis et al., 1999; Graef et al., 2001). TCR/CD28-costimulated c-*rel*$^{-/-}$ T cells exhibit impaired G1/S phase progression resulting from reduced IL-2 transcription (Kontgen et al., 1995). This defect is mitogen dependent, as it is largely overcome by PMA plus ionomycin stimulation. T cells lacking NF-κB1 exhibit a modest proliferative defect that is not rescued by provision of IL-2 (Zheng et al., 2001). Loss of RelA is reported to slightly diminish T cell proliferation (Zheng et al., 2001). Due to functional overlap among these proteins, essential roles ascribed to a particular Rel/NF-κB protein determined from the analysis of the corresponding mutant mouse is likely to represent only part of its overall function during T cell activation (Gerondakis et al., 1999). Limited analysis of certain compound Rel/NF-κB null mutants has indeed established redundant roles for these transcription factors in the T cell lineage (Grossman et al., 2000; Zheng et al., 2003). However, unlike the essential role for Rel/NF-κB during B cell ontogeny

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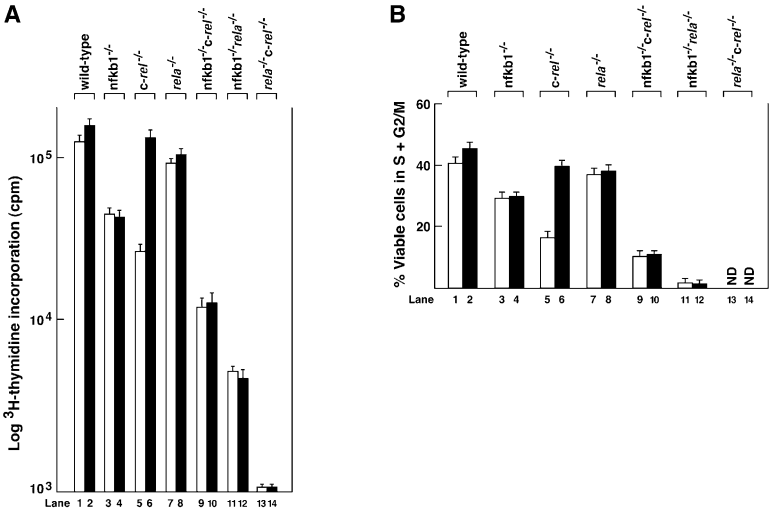

Figure 1. T Cell Division Is Dependent on the Rel/NF-κB Pathway

Purified wt, *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and c-*rel*$^{-/-}$*rela*$^{-/-}$ T cells were stimulated for 48 hr with anti-CD3/anti-CD28 mAbs in the absence (odd-numbered lanes) or presence (even-numbered lanes) of exogeneous IL-2.

(A) Cellular proliferation. T cell proliferation was measured by [^{3}H]thymidine incorporation.

(B) Cell cycle analysis. The fraction of viable T cells in the S, G2, or M phases of the cell cycle. Less than 1% of cells were in S or G2/M prior to stimulation.

(Gugasyan et al., 2000), loss of Rel/NF-κB function does not block the development of peripheral T cells (Hettmann et al., 1999). A similar theme of specific and redundant roles for the different NFAT family members during T cell activation has emerged from the study of mutant mice (Peng et al., 2001; Rengarajan et al., 2002). To determine what new roles different Rel/NF-κB proteins might serve early in T cell activation and how this function is regulated, we examined mature T cells lacking various combinations of NF-κB1, RelA, and c-Rel. Here we show that c-Rel and RelA are essential for the c-Myc-dependent growth of mitogen-activated T cells and that induced c-*rel* transcription, which preceeds c-*myc* expression, is regulated by a PKCθ-NFAT-dependent pathway.

Results

Redundant Roles for Rel/NF-κB during Mitogen-Induced T Cell Activation

To gain insight into what roles Rel/NF-κB transcription factors serve during T cell activation, T cells lacking NF-κB1, c-Rel, RelA, or combinations of these proteins were stimulated in culture with anti-CD3/anti-CD28 antibodies (Abs) in the absence or presence of exogenous IL-2 over 48 hr. Splenic T cells were isolated from *rag-1*$^{-/-}$ mice engrafted with wild-type (wt), *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, or *rela*$^{-/-}$c-*rel*$^{-/-}$ E12 fetal liver hemopoietic progenitors. This approach was adopted to ensure that the mitogenic response of Rel/NF-κB mutant T cells would not be influenced by environmental differences during development (Horwitz et al., 1997; Senftleben et al., 2001). Proliferation was monitored by tritiated thymidine incorporation (Figure 1A). The absence of RelA, as expected (Zheng et al., 2001), had a minimal effect on proliferation (lanes 7 and 8). While impaired c-*rel*$^{-/-}$ T cell proliferation (lane 5; Kontgen et al., 1995) was rescued by IL-2 (lane 6), the modest reduction in *nfkb1*$^{-/-}$ T cell proliferation was IL-2 independent (lanes 3 and 4). These defects were more pronounced in the absence of multiple Rel/NF-κB proteins. *nfkb1*$^{-/-}$c-*rel*$^{-/-}$ T cell proliferation (lane 9) was ~3- and 5-fold lower, respectively, than that of c-*rel*$^{-/-}$ and *nfkb1*$^{-/-}$ T cells, while *nfkb1*$^{-/-}$*rela*$^{-/-}$ T cell proliferation (lane 11) was ~10- and 30-fold less than *nfkb1*$^{-/-}$ and *rela*$^{-/-}$ T cells. The most severe defect resulted from a combined loss of c-Rel and RelA, with proliferation at levels only marginally higher than that observed for T cells in the absence of mitogen (lanes 13 and 14). Consistent with these trends, the frequency of cells in the S and G2/M phases of the cell cycle (Figure 1B) was markedly reduced in mitogen-stimulated *nfkb1*$^{-/-}$c-*rel*$^{-/-}$ (lanes 9 and 10) and *nfkb1*$^{-/-}$*rela*$^{-/-}$ (lanes 11 and 12) T cells (8% and 2%, respectively, compared with ~40% in activated wt cells). No viable *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells were detected in S or G2/M (lanes 13 and 14).

Mitogen-Induced T Cell Growth Is Blocked in the Combined Absence of RelA and c-Rel

The failure of activated *nfkb1*$^{-/-}$c-*rel*$^{-/-}$ B cells to enter S phase due to an inability to grow (Grumont et al., 2002) prompted an examination of mitogen-induced growth by Rel/NF-κB-deficient T cells. T cells were stimulated in culture with anti-CD3/anti-CD28 Abs plus IL-2 and growth measured after 24 hr by flow cytometry using forward light scatter analysis. A typical set of data is shown in Figure 2A. Despite a reduced frequency of mitogen-stimulated *nfkb1*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, and *nfkb1*$^{-/-}$*rela*$^{-/-}$ T cells entering S phase, their growth together with that of c-*rel*$^{-/-}$ and *rela*$^{-/-}$ T cells appeared normal. In contrast, *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells failed to undergo mitogen-induced growth. A growth block was also observed after PMA plus ionomycin treatment (results not shown), ruling out a stimulus-specific defect. Next, we examined if the failure of *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells to increase in size was associated with impaired RNA and protein synthesis. Transcription, measured by ^{3}H-uridine incorporation after the initial 12 hr period of stimulation with anti-CD3/anti-CD28 Abs (Figure 2B), established that *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells did not increase RNA synthesis upon mitogenic activation. The protein content of mitogen-activated *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells also failed to increase above prestimulation levels (Figure 2C).

To ensure that the failure of mitogen-treated *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells to grow reflected a defect in activation and not development, a cDNA encoding RelA was expressed in *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells using retroviral-medi-

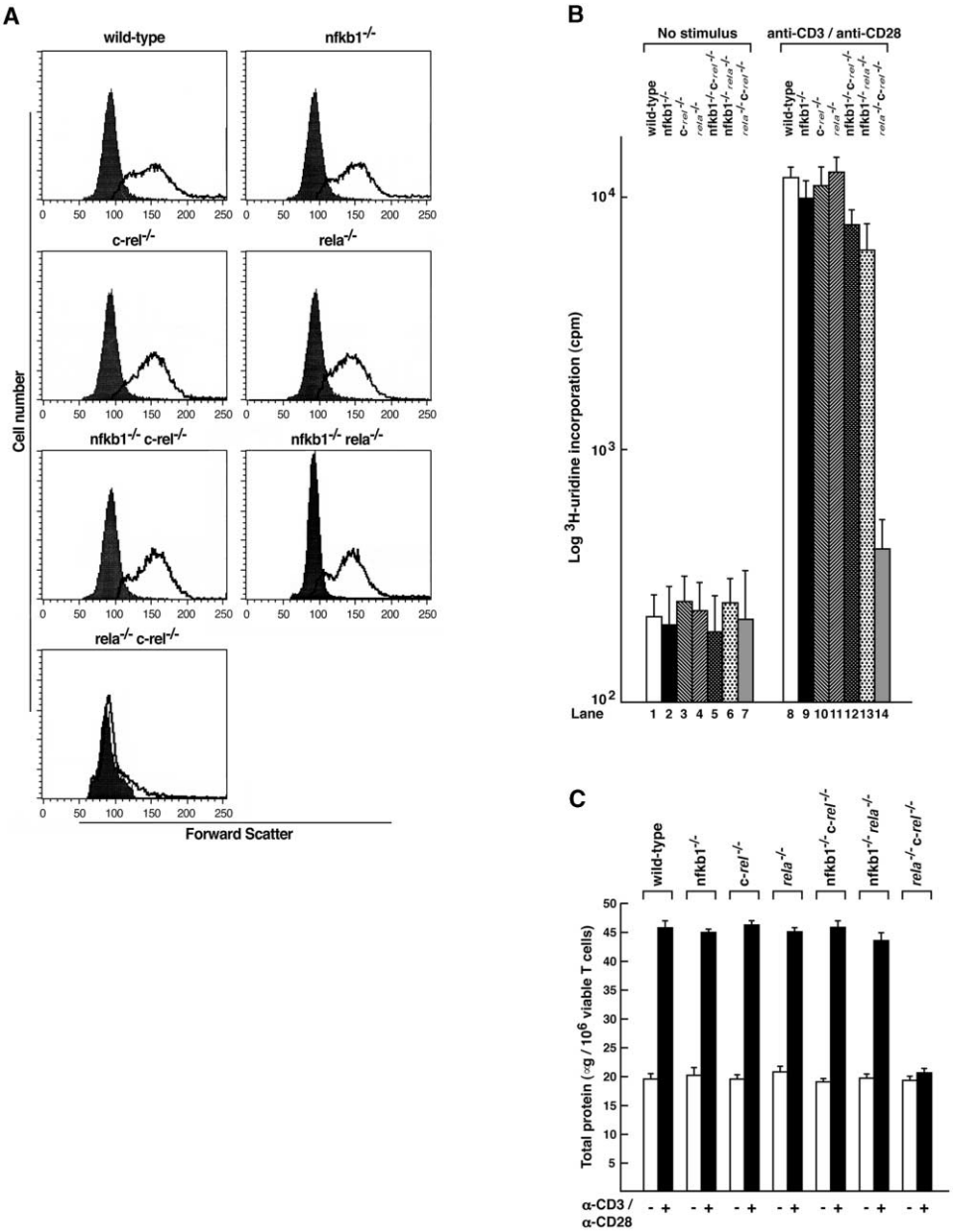

Figure 2. T Cell Growth Is Blocked in the Combined Absence of c-Rel and RelA

Quiescent Thy-1$^+$ wt, *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *rela*$^{-/-}$c-*rel*$^{-/-}$ splenic T cells untreated or activated in culture with anti-CD3/anti-CD28 mAbs plus IL-2.

(A) Mitogen-induced growth of T cells lacking different Rel/NF-κB proteins. After 24 hr, cells were examined by flow cytometry, with forward light scatter profiles (*x* axis) of untreated (closed) and activated (open) cells shown on a linear scale being a measure of cell size. The data are representative of three experiments.

(B) RNA synthesis in mitogen-activated T cells. RNA synthesis in wt (open bars), *nfkb1*$^{-/-}$ (closed bars), c-*rel*$^{-/-}$ (hatched bars), *rela*$^{-/-}$ (reverse hatched bars), *nfkb1*$^{-/-}$c-*rel*$^{-/-}$ (cross-hatched bars), *nfkb1*$^{-/-}$*rela*$^{-/-}$ (stippled bars), or *rela*$^{-/-}$c-*rel*$^{-/-}$ (gray bars) T cells that were untreated or stimulated for 18 hr was measured by [^{3}H]uridine incorporation. The data represents the mean ± SD of three experiments.

(C) Protein levels in mitogen-treated T cells. Cellular protein levels were measured in equivalent numbers of viable purified wt, *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, or *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells left untreated (open) or stimulated (closed) for 24 hr. The results represent the mean ± SD of three experiments.

ated gene transfer. *rag-1*$^{-/-}$ mice were engrafted with E12 *rela*$^{-/-}$c-*rel*$^{-/-}$ fetal liver hemopoietic progenitors infected with MY-EGFP (control) or MY-EGFPrela virus. Western blotting of Thy-1$^+$GFP$^+$ T cells from both groups of reconstituted mice confirmed that RelA was now expressed in T cells arising from progenitors infected with MY-EGFPrela (Figure 3A). Consistent with the redundant regulation of T cell growth by RelA and c-Rel, *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells transduced with MY-EGFPrela but not pMY-EGFP now increased in size when activated (Figure 3B).

We also noted that elevated levels of apoptosis accompanied the reduced proliferation of compound Rel/NF-κB mutant T cells (Supplemental Figure S1 at http://

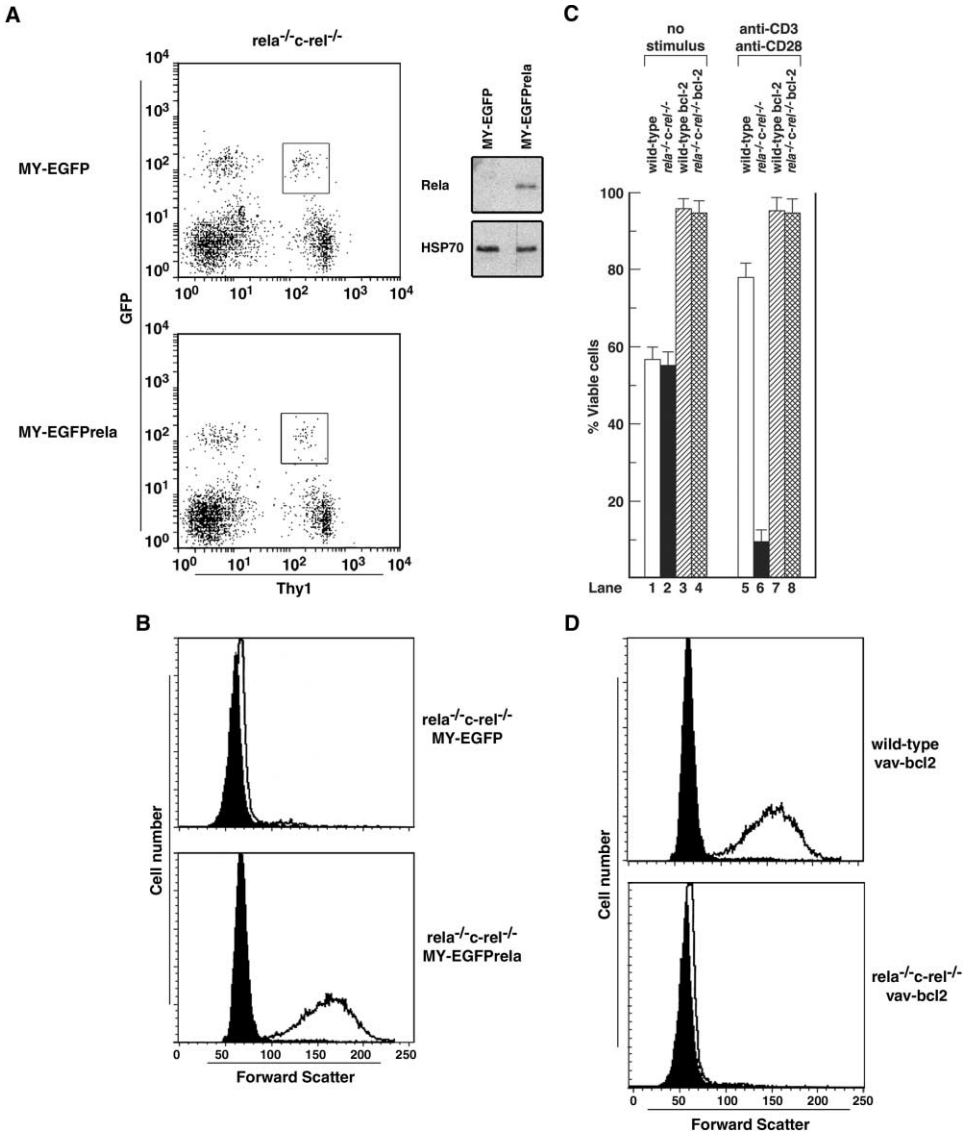

Figure 3. Increased Apoptosis in the Absence of RelA and c-Rel Does Not Influence T Cell Growth

(A) *rela* transgene expression in T cells. Equivalent numbers of GFP^{hi}Thy1^{+} splenic T cells (boxed) isolated by FACS from *rag-1*$^{-/-}$ mice reconstituted with MY-EGFP or MY-EGFPrela virus-infected *rela*$^{-/-}$c-*rel*$^{-/-}$ hemopoietic progenitors were subjected to Western blotting using anti-RelA and anti-HSP70 antibodies.

(B) *rela* transgene expression rescues the *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell growth defect. Forward light scatter profiles of MY-EGFP- or MY-EGFPrela-infected *rela*$^{-/-}$c-*rel*$^{-/-}$ GFP^{hi}Thy-1^{+} splenic T cells cultured for 24 hr without (closed) and with (open) anti-CD3/anti-CD28 Ab stimulation.

(C) Mitogen-induced death of *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells is inhibited by Bcl-2. The effect of transgenic Bcl-2 expression on the viability of wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells that were untreated or activated in culture for 24 hr; more than 99% of cells were viable at T0.

(D) Bcl-2 fails to rescue the *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell growth defect. Forward light scatter profiles of wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells expressing transgenic Bcl-2, 24 hr after culture without (closed) or in response to (open) anti-CD3/anti-CD28 Ab activation. All results are representative of two independent experiments.

www.immunity.com/cgi/content/full/21/1/19/DC1). To rule out that the failure of activated *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells to grow was not simply a consequence of these cells being destined to undergo apoptosis, a *bcl-2* transgene was introduced onto the *rela*$^{-/-}$c-*rel*$^{-/-}$ genetic background (Grossmann et al., 2000). Figure 3C shows that while passive cell death of wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells after 24 hr in culture was equivalent (lanes 1 and 2), the elevated levels of apoptosis in activated *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell cultures (24 hr; lane 6) was inhibited by Bcl-2 (lane 8). Importantly, despite Bcl-2 inhibiting mitogen-induced *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell death, growth was still blocked (Figure 3D), indicating that T cell growth and survival in response to mitogenic signals are independently regulated by RelA and c-Rel.

The Defect in Mitogen-Induced *rela*$^{-/-}$c-*rel*$^{-/-}$ T Cell Growth Is Due to Impaired c-*myc* Expression

Since the block in *nfkb1*$^{-/-}$c-*rel*$^{-/-}$ B cell growth is a direct consequence of reduced c-*myc* transcription

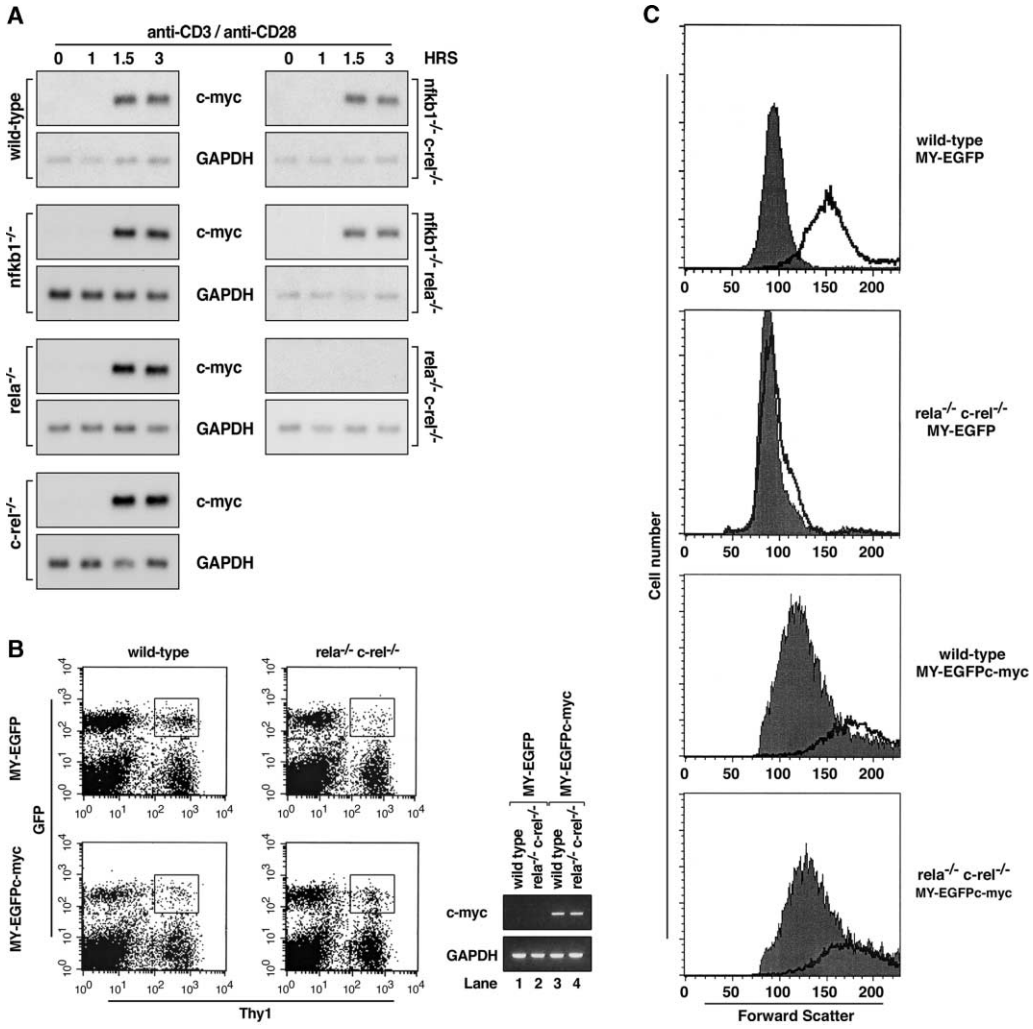

Figure 4. The Failure of Activated *rela*$^{-/-}$c-*rel*$^{-/-}$ T Cells to Grow Is Due to Impaired c-*myc* Expression

(A) Mitogen-induced c-*myc* expression is markedly reduced in *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells. 10 μg samples of total RNA isolated from wt, *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, or *rela*$^{-/-}$c-*rel*$^{-/-}$ splenic T cells prior to stimulation or after anti-CD3/anti-CD28 Ab activation for 1, 1.5, or 3 hr were subjected to Northern blot analysis. Filters were hybridized with c-*myc* and GAPDH cDNA probes. The data are representative of three experiments.

(B) c-*myc* transgene expression in T cells. c-*myc* transgene and GAPDH expression was determined by semiquantitative RT-PCR performed on RNA from equivalent numbers (5×10^4) of GFP^{hi}Thy1$^+$ splenic cells (boxed) isolated from *rag-1*$^{-/-}$ mice reconstituted with MY-EGFP or MY-EGFPc-myc virus-infected wt or *rela*$^{-/-}$c-*rel*$^{-/-}$ hemopoietic progenitors. The data are representative of four experiments.

(C) c-*myc* transgene expression rescues the *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell growth defect. Forward light scatter profiles of MY-EGFP or MY-EGFPc-myc virus-infected wt or *rela*$^{-/-}$c-*rel*$^{-/-}$ splenic T cells 24 hr after culture without (closed) or in response to anti-CD3/anti-CD28 Ab activation (open). The data are representative of three experiments.

(Grumont et al., 2002), we examined c-*myc* expression in mitogen-activated *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells (Figure 4A). c-*myc* mRNA levels, undetectable in resting wt T cells (lane 1), increased markedly within ~90 min of stimulation (lane 3) and were maintained at elevated levels for at least a further 90 min (lane 4). Whereas normal patterns of c-*myc* expression were observed in resting and activated *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, and *nfkb1*$^{-/-}$*rela*$^{-/-}$ T cells, c-*myc* mRNA levels in mitogen-treated *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells were not induced above that observed in quiescent cells.

To determine if the failure to upregulate c-*myc* expression accounted for the impaired growth of *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells, transgenic c-*myc* was expressed in mutant T cells. *rag-1*$^{-/-}$ mice were engrafted with wt or *rela*$^{-/-}$ c-*rel*$^{-/-}$ E12 fetal liver hemopoietic progenitors infected with control (MY-EGFP) or c-*myc*-expressing (MY-EGFPc-myc) murine retroviruses. GFP$^+$ splenic T cells were isolated by flow cytometry ~8 weeks after engraftment, and transgenic c-*myc* expression was examined in Thy-1$^+$GFP$^+$ wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ splenic T cells by semiquantitative RT-PCR using primers specific for retrovirus-encoded c-*myc*. Similar levels of transgenic c-*myc* mRNA were detected in both wt and *rela*$^{-/-}$ c-*rel*$^{-/-}$ T cells infected with MY-EGFPc-myc (Figure 4B). The effect of transgenic c-*myc* expression on T cell growth is shown in Figure 4C. In the absence of mitogen, wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells infected with MY-EGFPc-

myc, although equivalent in size, were larger than unstimulated cells of either genotype infected with MY-EGFP. Unstimulated MY-EGFPc-myc-infected cells also exhibited greater size heterogeneity than infected controls. As the degree of cell growth is influenced by the level of c-*myc* expression (Grumont et al., 2002), this size heterogeneity most likely reflects clonal variation in transgenic c-*myc* levels. Upon activation, wt but not *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells infected with MY-EGFP increased in size. During a 24 hr period of activation, both MY-EGFPc-myc-infected wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells underwent an additional, equivalent increase in growth beyond that seen in these cells prior to stimulation. This extra growth may be due in part to increased levels of c-Myc protein resulting from a mitogen-dependent increase in c-Myc stability (Grumont et al., 2002). The finding that transgenic c-*myc* expression was sufficient to overcome the block in mitogen-dependent *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell growth are consistent with a growth defect arising from the inability to upregulate c-*myc* expression.

Pro-T Cell Growth and Division Is Normal in the Absence of c-Rel and RelA

We had previously shown that thymocyte and peripheral T cell numbers are reduced in the combined absence of RelA and c-Rel (Grossman et al., 2000). Extensive proliferation normally occurs within the CD25$^-$CD44$^-$ (stage IV) population of double-negative (DN) pro-T cells. Given the fact that Rel/NF-κB induction by pre-TCR signals promotes the transition from stage III (CD25$^-$CD44$^+$) to stage IV (CD25$^-$CD44$^-$) (Voll et al., 2000), a developmental phase during which pro-T cell growth and division is c-Myc dependent (Douglas et al., 2001; Iritani et al., 2002), we examined *rela*$^{-/-}$c-*rel*$^{-/-}$ thymocyte growth and proliferation. Stage III and stage IV DN (CD4$^-$CD8$^-$HSA^{hi}) thymocytes were isolated from *rag-1*$^{-/-}$ mice engrafted with control (wt, c-*rel*$^{-/-}$, *rela*$^{-/-}$, or c-*rel*$^{-/-}$*rela*$^{+/-}$) or *rela*$^{-/-}$c-*rel*$^{-/-}$ E12 fetal liver hemopoietic progenitors. A block in *rag-1*$^{-/-}$ thymocyte development at stage III permits the unambiguous identification of donor-derived stage IV DN thymocytes, whereas distinguishing between stage III DN thymocytes of donor and host cell origin was not possible. Forward scatter analysis (Figure 5A) revealed that the median size of control and *rela*$^{-/-}$c-*rel*$^{-/-}$ stage IV thymocytes was equivalent and (as expected) larger than stage III cells. Cell cycle analysis (Figure 5B) demonstrated that the frequency of wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ stage IV cells in S or G2/M was similar, with more cell division occurring during stage IV. Consistent with the normal growth and division of *rela*$^{-/-}$c-*rel*$^{-/-}$ stage IV DN thymocytes, the levels of c-*myc* mRNA measured by real-time RT-PCR were comparable in control and mutant CD25$^-$CD44$^-$ thymocytes (Figure 5C). These findings establish that in pro-T cells, c-Rel and RelA are dispensible for growth, division, and c-*myc* expression.

In Mature T Cells, Mitogen-Induced c-*rel* Expression Is NFAT Dependent

Despite redundant roles for RelA and c-Rel in the control of mitogen-induced c-*myc* expression and T cell growth, the kinetics of c-*myc* mRNA induction coincided with the nuclear expression of c-Rel, not RelA. Given that the increased nuclear levels of c-Rel in T cells appeared to result from induced c-*rel* transcription, the temporal association between the mitogenic induction of c-*rel* and c-*myc* expression prompted an examination of the pathway regulating c-*rel* expression in activated T cells and its links to growth.

First, the potential involvement of RelA in mitogen-induced c-*rel* transcription was determined. Although the nuclear translocation of pre-existing NF-κB1/RelA precedes the induction of c-*rel* mRNA expression in activated T cells (Rao et al., 2003) and constitutive c-*rel* transcription in B cells is dependent on Rel/NF-κB regulation (Grumont et al., 1993), induced c-*rel* mRNA expression in anti-CD3/anti-CD28 Ab-activated *nfkb1*$^{-/-}$*rela*$^{-/-}$ splenic T cells was normal (Figure 6A). This ruled out a requirement for NF-κB in c-*rel* transcription.

The finding that the immunosuppressant FK506, an inhibitor of calcineurin (Rao et al., 1997), blocked induced c-*rel* mRNA expression in activated T cells (Venkataraman et al., 1995) raised the possibility that c-*rel* transcription in T cells was regulated by an NFAT-dependent pathway. Examination of the murine c-*rel* gene identified a putative NFAT binding site (5′-TGGAAA GAGCTATGCCA-3′), designated NFAT-R, 2305 nucleotides upstream of the major start site of transcription (Figure 6B). Like bona fide NFAT elements, which comprise composite binding sites for NFATC and AP-1 or CREB/ATF proteins, NFAT-R perfectly matched the consensus NFATC site and shared four of seven residues with the AP-1 consensus motif (Rao et al., 1997). To determine if NFAT-R was important for mitogen-induced c-*rel* transcription, c-*rel* promoter-reporter constructs with NFAT-R intact (pRel-luc) or altered (pRel$_m$-luc) to prevent NFAT binding were transfected into Jurkat T cells, which were then treated with PMA plus ionomycin, in the absence or presence of FK506 (Figure 6B). Whereas PMA/ionomycin, a potent NFAT activator, significantly upregulated pRel-luc transcription (lanes 5 and 6), this stimulus failed to activate pRel$_m$-luc (lane 12). Consistent with PMA/ionomycin-dependent induction of pRel-luc acting via NFAT, FK506 blocked the induced transcription of pRel-luc (lane 9).

The nuclear complexes that bound NFAT-R were examined by electrophoretic mobility shift assays (Figure 6C). In quiescent T cells (lane 1), two complexes, C1 and C2, bound a probe encompassing NFAT-R; C1 binding was highly reproducible but that of C2 was variable. In response to anti-CD3/anti-CD28 Abs or PMA and ionomycin (not shown), a novel complex of slower mobility (C3) induced within ~30 min bound the NFAT-R probe (lane 2). Binding of C2 and C3 to NFAT-R was dependent on the NFATc consensus motif, as the NFAT-R$_m$ probe, in which conserved residues essential for NFATC binding were mutated, failed to bind either complex (lanes 3 and 4). Consistent with the mitogen-induced C3 complex being dependent on NFAT activity, C3 binding to the NFAT-R probe was inhibited by FK506 (Figure 6D). The finding that NFATC1-specific antibodies supershifted the C3 complex (Figure 6E) or inhibited binding of C3 to NFAT-R (Supplemental Figure S2) confirmed that NFATC1 was indeed a component of this complex. The identity of the protein(s) in C2 remains unknown. Collectively, these data indicate that the mitogenic induction

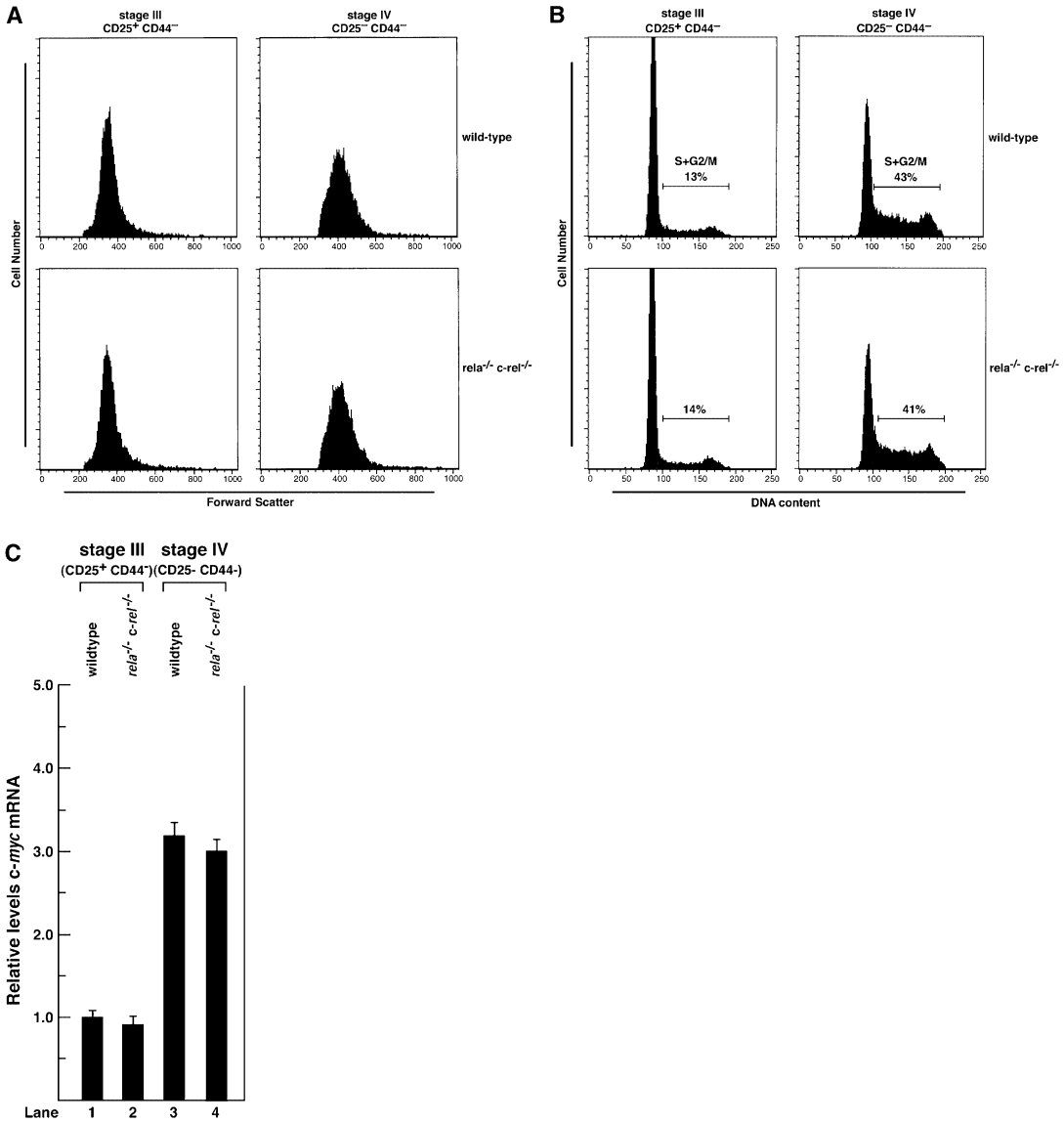

Figure 5. Growth and c-*myc* Expression Are Normal in *rela*$^{-/-}$c-*rel*$^{-/-}$ Thymocytes

(A) The size of *rela*$^{-/-}$c-*rel*$^{-/-}$ DN thymocytes is normal. Forward light scatter profiles of viable wt or *rela*$^{-/-}$c-*rel*$^{-/-}$ CD25^{+}CD44^{-} and CD25^{-}CD44^{-} DN thymocytes.

(B) Cell cycle analysis of *rela*$^{-/-}$c-*rel*$^{-/-}$ DN thymocytes. The fraction of viable CD25^{+}CD44^{-} and CD25^{-}CD44^{-} wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ DN thymocytes in G0/G1 or S/G2 plus M. The data in (A) and (B) are each representative of two experiments.

(C) c-*myc* expression in DN thymocytes. c-*myc* expression in wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ CD25^{+}CD44^{-} and CD25^{-}CD44^{-} DN thymocytes was assessed by real-time RT-PCR. c-*myc* mRNA expression in wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ CD25^{+}CD44^{-} (stage III) and CD25^{-}CD44^{-} (stage IV) cells is represented as relative to levels observed in wt cells. c-*myc* levels in each RNA sample have been normalized relative to GAPDH mRNA expression. The data represent the mean ± SD of three experiments.

of c-*rel* transcription in T cells is directly controlled by NFAT.

PKCθ Is Required for Mitogen-Induced T Cell Growth

Among the intracellular signaling molecules important for T cell proliferation induced by TCR and CD28 engagement that are essential for the activation of the NF-κB and NFAT/AP1 pathways is the calcium-independent PKC isoform, PKCθ (Isakov and Altman, 2002). Given the redundant roles of RelA and c-Rel in c-*myc*-dependent T cell growth, coupled with NFAT regulation of c-*rel* expression in T cells, pharmacological inhibitors of PKCθ (rottlerin) and NFAT (FK506) function were employed to determine the relationship between PKCθ, NFAT, RelA, c-*rel*, and c-*myc* in the control of mitogen-induced T cell growth. First, the consequences of inhibiting PKCθ or NFAT for T cell growth were examined (Figure 6A). Whereas FK506 at a concentration sufficient to inhibit the calcineurin-dependent activation of NFAT (Woo et al., 1990) did not block mitogenic growth, in the presence of rottlerin, mitogen-stimulated T cells failed to increase in size. This finding prompted us to examine if rottlerin inhibition of T cell growth was linked to im-

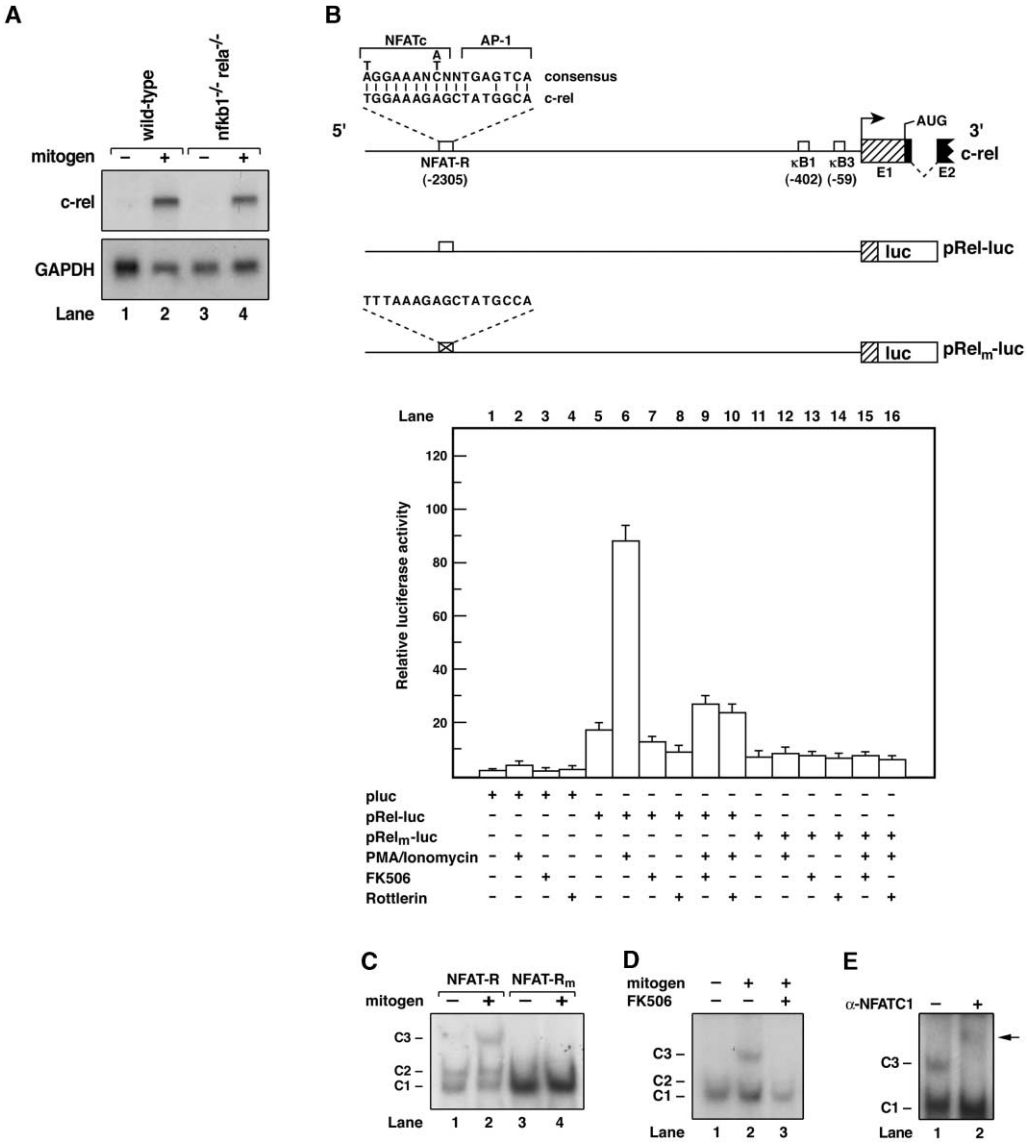

Figure 6. In T Cells, Mitogen-Induced c-*rel* Transcription Is Directly Regulated by NFAT

(A) c-*rel* expression in T cells is NF-κB independent. 5 μg samples of total RNA from wt or *nfkb1*$^{-/-}$*rela*$^{-/-}$ T cells prior to or after anti-CD3/anti-CD28 Ab activation for 1 hr was subjected to Northern blot analysis. Filters were hybridized with c-*rel* and GAPDH cDNA probes. The data shown are representative of two experiments.

(B) Analysis of the murine c-*rel* promoter. Shown is a schematic diagram of the c-*rel* 5′ flanking region and luciferase reporter plasmids. E1 and E2 correspond to exons; the hatched and closed regions of c-*rel* represent 5′ untranslated and coding regions, respectively. The arrow designates the major start site of transcription. Open boxes represent the κB sites and putative NFAT element (5′-TGGAAAGAGCTATGCCA-3′); the corresponding symbol with a cross represents the mutated NFAT motif (5′-TTTAAAGAGCTATGTCA-3′). The luciferase gene, luc, is depicted as an open box. Jurkat T cells were transfected with reporter plasmids, and 24 hr later, cells were left untreated or stimulated for 6 hr with PMA/ionomycin, FK506, rottlerin, PMA/ionomycin/FK506, or PMA/ionomycin/rottlerin. Promoter activity is displayed as relative luciferase units. The results represent the mean luciferase activity ± SD from four sets of transfections.

(C) A mitogen-induced complex binds NFAT-R. Nuclear extracts from resting or anti-CD3/anti-CD28 Ab-activated (30 min) T cells were incubated with radiolabeled NFAT-R or NFAT-R_m probes, then resolved on nondenaturing acrylamide gels. Shown are the inducible slow mobility (C3) and constitutive fast mobility complexes (C1 and C2).

(D) FK506 inhibits the mitogen-inducible complex. Nuclear extracts from T cells that were resting or mitogen activated in the absence or presence of FK506 were incubated with radiolabeled NFAT-R probe.

(E) The inducible C3 complex contains NFATC1. Nuclear extracts from activated T cells were incubated with irrelevant (lane 1) or NFATC1 (lane 2) specific sera. The supershifted C3 complex is indicated by the arrow.

paired c-*myc* expression (Figure 7B). While the upregulation of c-*myc* in activated T cells was not affected by FK506 (lane 3), rottlerin suppressed the induction of c-*myc* (lane 4). The capacity of rottlerin to mimic the block in T cell growth observed in the combined absence of c-Rel and RelA suggested a mechanism involving the inhibition of both these transcription factors. Consistent with PKCθ being necessary for RelA nuclear import (Sun

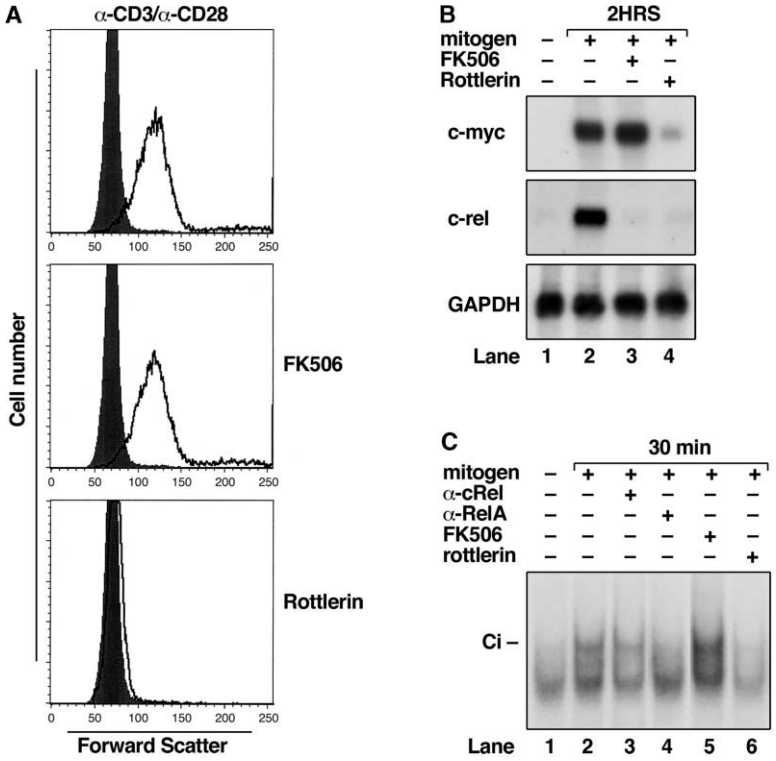

Figure 7. c-*myc* Dependent T Cell Growth Is Regulated by PKCθ

(A) PKCθ inhibitor rottlerin blocks T cell growth. Splenic T cells were untreated or stimulated for 24 hr with anti-CD3/anti-CD28 Abs alone or in combination with FK506 or rottlerin. Shown are forward light scatter profiles of viable unstimulated (closed) or activated (open) T cells (*x* axis). The data are representative of three experiments.

(B) Rottlerin blocks mitogen-induced c-*myc* expression. Total RNA isolated from wt T cells that were untreated or stimulated (2 hr) with anti-CD3/anti-CD28 Abs in the absence (lane 2) or presence of FK506 (lane 3) or rottlerin (lane 4) was subjected to Northern blot analysis. Filters were sequentially hybridized with c-*myc*, c-*rel*, and GAPDH probes.

(C) Rottlerin, but not FK506, inhibits the nuclear import of RelA. Nuclear extracts from wt T cells that were untreated or activated for 30 min with anti-CD3/anti-CD28 Abs in the absence (lanes 2–4) or presence of FK506 (lane 5) or rottlerin (lane 6). c-Rel- and RelA-specific Abs were pre-incubated with nuclear extract from activated T cells prior to adding radiolabeled κB probe. The mitogen-induced complex is designated Ci.

et al., 2000), a nuclear complex (Ci) induced in T cells within 30 min of activation (Figure 7C, lane 2) containing RelA (Figure 7C, lane 4) but not c-Rel (lane 3) was inhibited by rottlerin (lane 6). Furthermore, rottlerin prevented the mitogen-induced expression of c-*rel* mRNA (Figure 7B, lane 4) and inhibited PMA plus ionomycin-mediated transcription of pRel-luc (Figure 6B, lane 10). In contrast to the action of rottlerin, FK506, while inhibiting c-*rel* expression (Figure 7B, lane 3), did not prevent the nuclear induction of RelA complexes (Figure 7C, lane 5). These results are consistent with PKCθ being able to control c-*myc*-dependent T cell growth by regulating the nuclear translocation of RelA- and NFAT-mediated c-*rel* transcription.

Discussion

Prominent in the control of gene expression during the early stage of T cell activation are the NFAT and Rel/NF-κB transcription factors. A detailed understanding of the precise physiological roles served by the Rel/NF-κB transcription factors in this process is complicated by functional redundancy (Gerondakis et al., 1999) and temporal changes in nuclear expression of different Rel/NF-κB dimers (Molitor et al., 1990). Using T cells that lack different combinations of the major Rel/NF-κB factors normally activated in the response to mitogens, we show that c-*myc*-dependent T cell growth is controlled by RelA and c-Rel via PKC- and NFAT-dependent pathways that coordinate RelA nuclear import and induced c-*rel* transcription.

A body of evidence gathered from different experimental systems indicate that c-Myc regulates the mitogenic induction of cell growth (Eisenman, 2001; Iritani et al., 2002). Here, we extend this link to include mitogen-activated T cells. While our findings are consistent with endogenous c-Myc inducing growth in primary B cells (Morena de Alboran et al., 2001; Grumont et al., 2002) and thymocytes (Douglas et al., 2001), it contradicts a report (Trumpp et al., 2001) that concluded that in T cells c-Myc is dispensible for growth but instead regulates cell cycle entry in response to mitogenic signals. Utilizing a mouse strain that expressed a single functional loxP-targeted c-*myc* allele and carried a Mx-Cre transgene, T cells lacking c-Myc were generated by in vivo administration of pIC (Trumpp et al., 2001). It was noted, however, that this inducible deletion of c-*myc* was incomplete, with 28%–48% of T cells isolated from these pIC-treated mice retaining the functional c-*myc* allele. Given that an absence of c-Myc increases T cell death during mitogen stimulation (Trumpp et al., 2001), a finding consistent with high levels of apoptosis seen during *rela*$^{-/-}$c-*rel*$^{-/-}$ T cell activation (Figure 3C), the apparent growth of mitogen-stimulated c-*myc* null T cells observed in the study by Trumpp may instead reflect the preferential survival of T cells retaining a functional c-*myc* allele.

The findings presented here for T cells, together with a report on B cell growth (Grumont et al., 2002), establish that the Rel/NF-κB pathway serves a common role controlling c-*myc*-dependent growth of mitogen-activated lymphocytes. In the T lineage, the link between RelA/c-Rel, c-*myc*, and growth appears to be restricted to mature T cells, with cell growth, division, and c-*myc* expression found to be normal in *rela*$^{-/-}$c-*rel*$^{-/-}$ thymocytes. Moreover, normal growth of *nfkb1*$^{-/-}$*rela*$^{-/-}$ and *nfkb1*$^{-/-}$c-*rel*$^{-/-}$ DN thymocytes (results not shown) indicates that this function may be Rel/NF-κB independent.

Given the inhibition of Rel/NF-κB function increases $CD25^{-}CD44^{-}$ DN thymocyte apoptosis (Voll et al., 2000), this indicates that the role of this pathway in thymocyte homeostasis is one of cell survival rather than division.

Functional overlap between c-Rel and other Rel/NF-κB family members in controlling c-*myc*-induced growth differs between B and T cells. Whereas c-Rel and RelA are required for c-*myc* expression in T cells, c-Rel and NF-κB1 serve this role in B cells (Grumont et al., 2002). The reason for this lineage-specific difference remains to be determined but may be related to a hierarchy of importance among these transcription factors in B and T cells, a notion supported by a predominance of NF-κB1/c-Rel in mature B cells but not T cells (Gugasyan et al., 2000). Despite functional overlap between c-Rel and RelA in growth control, the kinetics of induced c-*myc* expression in T cells coincide with the nuclear expression of c-Rel, not RelA. Why then Rel/NF-κB-dependent T cell growth is not controlled by c-Rel alone is at present unclear. We have also found that enforced c-Myc expression, while able to overcome the block in growth afflicting *rela*$^{-/-}$c-*rel*$^{-/-}$ T cells, is insufficient to rescue the mitogen-induced proliferation of these mutant T cells (Supplemental Figure S3). This indicates that RelA and c-Rel serve functions in addition to promoting growth that are essential for T cell proliferation, akin to the multiple roles of Rel/NF-κB in B cell activation and proliferation (Grumont et al., 2002).

Our study shows that mitogen-induced c-*rel* transcription in T cells is regulated by a PKCθ/NFAT-dependent pathway. Although FK506 inhibition of c-*rel* expression in T cells (Venkataraman et al., 1995) had implicated NFAT in the control of c-*rel*, our identification of a consensus NFAT element in the murine c-*rel* promoter that bound NFATC1 and was required for c-*rel* transcription establishes c-*rel* as a direct transcriptional target of NFAT. Furthermore, we show that PKCθ, which is required for the expression of AP-1 (Pfeifhofer et al., 2003), a component of NFAT, was also necessary for c-*rel* expression. Even though PKCθ lies upstream of both the NF-κB and NFAT pathways, our findings indicate its role in c-*rel* transcription is mediated through NFAT. Importantly, our findings suggest that during the early stages of T cell activation, a regulatory hierarchy exists between NFAT and the c-Rel component of the NF-κB pathway, which is coordinated by PKCθ. The integration of these two signaling pathways through NFAT regulation of c-*rel* transcription may offer an additional mechanism for controlling the expression of genes such as IL-2 and GM-CSF, which are coregulated in T cells by these two families of transcription factors (Gerondakis et al., 1996; Rao et al., 1997, 2003).

Although mitogen-induced T cell growth is not blocked by concentrations of FK506 sufficient to inhibit c-*rel* expression, we noted that at a 10-fold greater concentration (5 μM), FK506 does block T cell growth. Importantly, this failure of T cells to grow was not associated with an inhibition of nuclear RelA or c-*myc* expression (R.G., unpublished results) and instead may be due to calcineurin-independent effects of FK506 (Halloran et al., 1998). Such a conclusion is consistent with T cell growth not being affected by an absence of various NFAT family members (Peng et al., 2001; Yoshida et al., 1998; Rengarajan et al., 2002). A role for NFAT in cell growth is, however, cell type specific, with cardiomyocyte growth blocked in the absence of NFATC2 (Horsley and Parlath, 2002). In contrast, PKCθ is essential for T cell growth. Although the inhibition of mitogen-induced growth and c-*myc* expression by rottlerin coincides with a combined block in RelA nuclear translocation and c-*rel* transcription, we cannot rule out that rottlerin inhibition of growth may involve PKC targets other than NFAT/AP-1 and NF-κB. Finally, the link established here between PKCθ and the c-Rel/RelA-dependent control of c-*myc*-mediated T cell growth may have implications for cancer therapy. Deregulated Rel/NF-κB signaling, a hallmark of many lymphoid cancers (Rayet and Gelinas, 1999), is likely to be important in cancer cell proliferation. Given that PKCθ expression is largely restricted to mature T cells (Isakov and Altman, 2002), our study indicates that targeting PKCθ function could disrupt the growth and division of T cell leukemias with high levels of Rel/NF-κB activity that display a mature T cell phenotype.

Experimental Procedures

Mice

c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, and *vav-bcl-2* mice on a C57BL6 background have been described (Grossmann et al., 2000; Grumont et al., 2002). The *rela*$^{+/-}$, *nfkb1*$^{-/-}$*rela*$^{+/-}$, and *rela*$^{+/-}$c-*rel*$^{-/-}$ parental strains used to generate *rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and c-*rel*$^{-/-}$*rela*$^{-/-}$ embryos were derived from mice backcrossed onto C57BL/6 (n = 9–10 backcross generations).

Fetal Liver Cell Engraftment

Splenic T lymphocytes of all genotypes (wt, *nfkb1*$^{-/-}$, c-*rel*$^{-/-}$, *rela*$^{-/-}$, *nfkb1*$^{-/-}$c-*rel*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, *rela*$^{-/-}$c-*rel*$^{-/-}$, *vav-bcl-2*, and *rela*$^{-/-}$c-*rel*$^{-/-}$*vav-bcl2*) were generated by engrafting *rag-1*$^{-/-}$ mice with E12 fetal liver hemopoietic progenitors as described (Grossmann et al., 2000).

Purification of T Cells and Thymocytes

Purified splenic T cells were obtained by cell sorting on a FACStar Plus (Becton Dickinson, CA) after staining with anti-Thy-1 antibodies. The purity of all sorted splenic T cell populations was >97%. Double-negative ($CD4^{-}CD8^{-}HSA^{hi}$) thymocytes purified from *rag-1*$^{-/-}$ mice reconstituted with control or *rela*$^{-/-}$c-*rel*$^{-/-}$ fetal liver hemopoietic progenitors first involved the depletion of unwanted cells by negative cell sorting (O'Reilly et al., 1997). The remaining cells were stained with CD25 and CD44 mAbs, then sorted into $CD25^{-}CD44^{+}$ (stage III) and $CD25^{-}CD44^{-}$ (stage IV) populations, the purity of which was >95%.

Cell Stimulation

Purified splenic T cells (5×10^5/ml) were stimulated with PMA (2 ng/ml) plus ionomycin (1 μg/ml) or plate bound anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) mAbs in the absence or presence of exogenous IL-2 (500 U/ml). FK506 and rottlerin (Calbiochem, La Jolla, CA) were added to cultures at final concentrations of 0.5 μM and 5 μM, respectively.

Flow Cytometric and Cell Cycle Analysis

Cultured T cells that were untreated or mitogen activated for 24 hr were stained with PI, and the forward light scatter profile of viable cells was determined by flow cytometry as described (Grumont et al., 2002). Cell division in purified thymocyte populations and in mitogen-stimulated T cell cultures was determined by assessing DNA content using PI staining (Grumont et al., 1998).

DNA, RNA, and Protein Synthesis Assays

T cells (3×10^5 cells/ml) cultured in 100 μl of medium (DMEM/10% FCS/50 μM βME) in 96-well microtiter plates were stimulated with mitogens and at various times DNA synthesis was measured by

adding 0.5 μCi of [^{3}H]thymidine for 6 hr. For RNA synthesis, cultures were pulsed 12 hr after mitogenic activation with 0.25 μCi of [^{3}H]uridine for an additional 6 hr. In both cases, incorporated radioactivity measured by scintillation counting. Cellular protein concentrations in T cells was determined by the Bradford assay.

Northern Blotting

10 μg samples of total RNA isolated from purified resting or activated splenic T cells were subjected to Northern blot analysis (Grumont et al., 2002). Probes were a mouse 1.4 kb Xho1 c-*myc* cDNA, a rat 1.1 kb Pst glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA, and a mouse 2.9 kb EcoR1 c-*rel* cDNA.

Western Blotting

Total cellular RelA levels were monitored in MY-EGFPrela virus-infected T cells (2×10^5/sample) by Western blotting (Grumont et al., 2002) using a RelA-specific antisera (Grumont and Gerondakis, 1994).

Plasmid Constructs

The plasmid pRel-luc comprised a 2.64 kb genomic fragment that encompassed the mouse c-*rel* 5′ untranslated sequence, transcription initiation sites, plus 2.39 kb of upstream sequence inserted into the promoterless luciferase reporter plasmid, pA3luc. The plasmid, pRel$_m$-luc, is a derivative of pRel-luc in which the NFAT-R binding site (5′-TGGAAAGAGCTATGCCA-3′) was altered by site-directed in vitro mutagenesis (5′-TTTAAAGAGCTATGCCA-3′). The retroviral expression vectors pMY-EGFPc-myc and pMY-EGFPrela were generated by inserting murine c-*myc* and rela cDNAs, respectively, into pMY-EGFP (Onai et al., 2000).

Electrophoretic Mobility Shift Assays

[^{32}P]dATP end-labeled probes corresponding to the mouse c-*rel* κB3 site (5′-GCGGGAAATCCCCC-3′) and the wild-type (5′-ATTACTGGA AAGAGCTATGCCACCCAA-3′) or mutant (5′-ATTACTTTAAAGAGCT ATGCCACCCAA-3′) NFAT-R element were incubated with ~2 μg of nuclear extract prepared from unstimulated or activated (30 min) T cells as described for NF-kB (Grumont and Gerondakis, 1994) and NFAT (Shang et al., 1999). Antibodies specific for mouse NFATC1 (Santa Cruz, SC-1149X), RelA, and c-Rel (Grumont and Gerondakis, 1994) were incubated with nuclear extracts on ice for 30 min before adding the radiolabeled probe. Reactions were then incubated for 20 min at room temperature, 2 μl of Ficoll dye were added, and the reactions were fractionated on 5% nondenaturing polyacrylamide gels. Gels were dried and exposed to autoradiography at −70°C.

Luciferase Assays

Jurkat T cells were transiently transfected by electroporating 10^7 cells (270 V, 975 μF) with equivalent amounts of DNA, and 24 hr later, cells were either left untreated or activated with PMA (20 ng/ml) plus ionomycin (1 μM) in the absence or presence of FK506 (0.5 μM) or Rottlerin (5 μM) for a further 6 hr, after which time luciferase assays were performed using a dual-luciferase reporter system (Promega). All results represent luciferase activities normalized against a control Renilla luciferase reporter, pRL-TK. The amounts of plasmids used were pRL-TK, 1 μg; pA3luc (pluc), 2 μg; pRel-luc, 2 μg; and pRel$_m$-luc, 2 μg.

Retrovirus Production and Infections

Helper virus free stocks of MY-EGFP, MY-EGFPc-myc, and MY-EGFPrela were generated by transient transfection of BOS23 cells, and fetal liver suspensions were infected with retroviruses using a spin infection procedure (Gugasyan et al., 2002). After three rounds of infection, between 10% and 20% of cultured cells were routinely GFP positive. 10^6 of these cultured hemopoietic cells were then engrafted into sublethally irradiated (300 Rad) *rag-1*$^{-/-}$ mice.

Reverse Transcription PCR

Total RNA was isolated from FACS-purified GFP$^+$Thy-1$^+$ wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ splenic T cells (2×10^5 cells/population) or CD25$^-$ CD44$^+$ and CD25$^-$CD44$^-$ double-negative wt and *rela*$^{-/-}$c-*rel*$^{-/-}$ thymocytes (5×10^5 cells/population). cDNA synthesis was performed using equivalent amounts of RNA as described (Grumont et al., 2001). For semiquantitative PCR, cDNA was first denatured at 94°C for 5 min, then amplification was performed for 25 cycles with each cycle programmed for denaturation at 94°C for 45 s, annealing at 58°C for 60 s, followed by elogation at 72°C for 90 s. Samples were then fractionated on a 1.0% agarose gel. The sequences of the oligonucleotides used for the amplification of β-actin and retrovirus-encoded c-*myc* were viral c-*myc* (forward, 5′-CAAAGTAGACGG CATCGCAGCTT-3′, pMY-EGFP vector; reverse, 5′-ATTTGGGC GAGCTGCTGTCGTTCA-3′, mouse c-*myc* exon 2; product size 717 bp) and β-actin (forward, 5′-CTGAAGTACCCATTGAACATGGC-3′; reverse, 5′-CAGAGCAGTAATCTCCTTCTGCAT-3′; product size 726 bp). Endogenous c-*myc* expression in double-negative thymocytes was measured using SYBR green real-time PCR reactions as described (Rao et al., 2003). c-*myc* mRNA levels were normalized by taking an aliquot of each sample and performing quantitiative PCR for GAPDH mRNA expression to compensate for input RNA variation and inefficiencies in cDNA synthesis. Primer sets used for real-time PCR were c-*myc* (forward, 5′-ACCAACAGGAACTATGACCTC-3′; reverse, 5′-AAGGACGTAGCGACCGCAAC-3′) and *GAPDH* (forward, 5′-TTCACCACCATGGAGAAGGC-3′; reverse, 5′-GGCATGGACTGT GGTCATGA-3′).

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