Myc stimulates B lymphocyte differentiation and amplifies calcium signaling

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Introduction

Members of the Myc family of nuclear protooncogenes are known to stimulate cell division and transformation. However, the roles of Myc in cell differentiation have been difficult to assess, in part because of the early embryonic lethality of N-myc– (Charron et al., 1992) and c-myc (Davis et al., 1993)–null mice and the difficulties in experimentally distinguishing proliferation from differentiation using in vitro approaches. In this study, we used B cell–specific deletion or overexpression of c- or N-myc to explore the roles of Myc in B lymphocyte development and lymphoma formation. B cell development in the bone marrow (BM) and fetal liver are marked by a series of cell fate decisions, which are controlled by checkpoints that ensure that all mature B lymphocytes are capable of producing functional antibodies (for review see Hendriks and Middendorp, 2004). Pluripotent hematopoietic progenitors initially become committed to the B lineage in response to growth factors and stromal cell interactions. The least mature committed B cell progenitors, called pre–B cells, have their Ig heavy chain (HC) and light chain (LC) genes in germline (GL) configuration. The Ig HC genes rearrange before the Ig LCs, and with successful in-frame DH-JH and then VH-DH-JH juxtaposition, the Ig HC appears at the cell surface along with the signal-transducing proteins Igα and Igβ and the surrogate LC α5 and Vpre-B1 and 2 as a complex called the pre–B cell receptor (BCR; termed large pre–B cells). The pre–BCR then signals large pre–B cells to proliferate, mature to the small pre–B cell stage, extinguish rearrangement of the other allelic Ig HC (a process called allelic exclusion), and initiate Ig LC transcription and rearrangement (Vκ and Vλ). Successful in-frame Ig Vλ1-Iκ rearrangement allows the Ig HC and LC proteins to pair and form IgM molecules on the surface of immature B cells, at which point they migrate from the BM to the periphery, where development continues.

Formation of the pre-BCR represents a critical checkpoint for ensuring that maturing B cells have in-frame VH-DH-Iκ rearrangements. Signaling is induced by pre-BCR aggregation in lipid rafts, resulting in activation of the Src family protein...
tyrosine kinases (PTKs) Blk, Lyn, and Fyn, which then phosphorylate tyrosine residues within Igα and Igβ. This results in recruitment and activation of the Syk PTK (and to a lesser extent ZAP70) and the adaptor SLP-65 (also known as BASH or BLNK). Phosphorylation of SLP-65 by Syk further activates multiple signaling pathways, including the Ras-Raf and phosphatidylinositol 3-kinase (PI3K) pathways, as well as the Tec family PTKs Btk and Tec, which, together with Syk, activate PLCγ, resulting in Ca2+ mobilization (see Fig. 8; for review see Hendriks and Middendorp, 2004). Altogether, these events result in expression and/or activation of a set of transcription factors including extracellular regulated kinase, nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB), and Myc.

Targeted deletion studies in mice have revealed essential roles for the majority of the described surface and cytoplasmic molecules in the development of pre-B cells (for review see Hendriks and Middendorp, 2004). However, the roles of nuclear factors such as Myc in B cell development remain unclear, in part because genetic redundancy by related transcription family members or lethality after tissue-wide deletion. The Myc family of basic helix-loop-helix transcription factors bind DNA sequences called E boxes (CACGTG) as a heterodimer with the related basic helix-loop-helix factor Max (for review see Eisenman, 2001). This results in initiation of transcription, although transcriptional repression by Myc has been noted, probably through inhibition of the Miz-1 transcription factor (Li et al., 1994; Staller et al., 2001). Of the three mammalian myc family members, only c- and N-myc are expressed in B lymphocytes. c-myc is initially expressed during the pro-B cell stage in response to cytokines, including IL-7 (Morrow et al., 1992; Dillon and Schlissel, 2002). After pre-BCR expression in large pre-B cells, both c- and N-myc are expressed during the maturation and expansion to small pre-B cells (Zimmerman and Alt, 1990). Thereafter, only c-myc is expressed in immature and mature B cells after B cell activation. In this study, we took a unique genetic approach in mice to show that Myc stimulates B cell differentiation and expansion downstream of the pre-BCR and Tec PTKs. Moreover, we uncovered a novel feature of Myc in amplifying [Ca2+]i, as a mechanism to concurrently stimulate the expression of both Myc and Ca2+-regulated genes, which are essential for both cell division and differentiation.

**Results**

**Mice deficient in Myc have impaired pre-B cell development**

To examine the roles of Myc in early B cell development and to overcome potential functional redundancy between c- and N-myc, we generated mice deficient in both N- and c-myc genes selectively in B cells using the Cre-loxP system (Sauer, 2002), c-myc<sup>fl/fl</sup> mice (de Alboran et al., 2001) were bred to N-myc<sup>fl/fl</sup> mice (Knoepfler et al., 2002), which also carried the CD19<sup>cre</sup> transgene (Tg), to generate N-myc<sup>fl/fl</sup> c-myc<sup>fl/fl</sup> CD19<sup>cre+</sup> mice. The CD19 promoter drives expression of the Cre recombinase only in B lineage cells throughout their development (Rickert et al., 1997). Semi-quantitative PCR (qPCR) analysis of sorted B220<sup>+</sup> CD43<sup>+</sup> and B220<sup>-</sup> CD43<sup>-</sup> B cell progenitors in the BM revealed ~75/90% deletion of c-myc and ~70/95% deletion of N-myc genes, respectively, in the presence of CD19<sup>cre</sup> (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200704173/DC1).

Total BM cells were isolated from mice deficient in c- and/or N-myc selectively in B cells, and B cell development was analyzed using flow cytometry (see Fig. 3 A; Hardy and Hayakawa, 2001). Decreasing c-myc expression alone (c-myc<sup>fl/fl</sup> CD19<sup>cre+</sup>) impairs the generation of B220<sup>+</sup> CD43<sup>+</sup> pre-B cells, whereas decreasing N-myc expression alone (N-myc<sup>fl/fl</sup> CD19<sup>cre+</sup>) does not consistently have any effect (unpublished data). However, depletion of total myc expression in N-myc<sup>fl/fl</sup> c-myc<sup>fl/fl</sup> CD19<sup>cre+</sup> mice results in maximal decreases in the percentage (Fig. 1 A) and number (Fig. 1 B) of B220<sup>+</sup> CD43<sup>+</sup>, B220<sup>+</sup>Ig<sup>H</sup> pre-B cells, immature B cells (B220<sup>+</sup>Ig<sup>M</sup>−), and mature B cells (B220<sup>+</sup>Ig<sup>M</sup>+) relative to CD19<sup>cre−</sup> mice. These results suggest that Myc is required for pre-B to pre-B cell maturation.

In mice, pre-B cells proliferate in response to cytokines such as IL-7 produced by BM stromal cells (Fleming and Paige, 2002). Because IL-7 signaling is known to stimulate the expression of c-myc, we examined the ability of B cell progenitors to divide in response to IL-7. Total BM cells from N-myc<sup>fl/fl</sup> c-myc<sup>fl/fl</sup> CD19<sup>cre+</sup> or CD19<sup>cre+</sup> mice were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in the presence or absence of IL-7 for 36 h, and the cell division profile of B cell progenitors was examined. As shown in Fig. 1 C, the majority of B cell progenitors from either N-myc<sup>fl/fl</sup> c-myc<sup>fl/fl</sup> CD19<sup>cre−</sup> or CD19<sup>cre+</sup> mice do not divide in the absence of IL-7. However, in response to IL-7, pre-B cells from CD19<sup>cre+</sup> mice undergo an average of four divisions, whereas cells from CD19<sup>cre−</sup> mice either do not divide at all or undergo several divisions, likely as a result of the loss of cells that contain the deleted c-myc alleles (Fig. S1 B; de Alboran et al., 2001).

To determine whether the reduction in myc levels affects the development of peripheral B cells, we purified splenocytes from N-myc<sup>fl/fl</sup> c-myc<sup>fl/fl</sup> CD19<sup>cre+</sup> and CD19<sup>cre−</sup> mice and characterized B cell subsets by flow cytometry. Splenic B cells are subdivided into additional stages of maturation and function, including transitional 1 and 2 (T1 and T2), follicular mature, and marginal zone (MZ; Hardy and Hayakawa, 2001). As shown in Fig. 2 A, CD19<sup>cre−</sup> mice exhibit a significant reduction in the number of all B cell subsets. This correlates with reduced serum IgM, IgG2a, and IgG1 in CD19<sup>cre+</sup> compared with CD19<sup>cre−</sup> mice after immunization with keyhole limpet hemocyanin (KLH; Fig. 2 C). These results suggest that Myc regulates final B cell numbers in part by regulating pre-B cell development and, potentially, peripheral B cell differentiation.

Peripheral B cells can be further classified as B2 conventional B cells, which reside in lymphoid tissues such as the spleen, and as B1 B cells, which represent the majority of B cells found in peritoneal and pleural cavities and are IgM<sup>+</sup>, CD5<sup>+</sup>, and CD23<sup>−</sup>. As shown in Fig. 2 B, deletion of c- and N-myc together results in a decrease in both CD5<sup>+</sup>IgM<sup>+</sup> peritoneal B1 B cells and CD5<sup>+</sup>IgM<sup>+</sup>B2 B cells. These results suggest that...
Myc is essential for the development of both B2 and B1 B cells and for establishing final B cell numbers.

Eμ-myc Tg stimulates the maturation and expansion of RAG2-null pro-B cells

It is unclear from the gene-targeting strategy whether the effects of Myc are exclusively in modulating the expansion of a population of cells that have already differentiated to the pre–B cell stage or whether Myc may also stimulate the differentiation of pre-BCR+ cells. To address this question, we took a classical genetic approach to determine whether Myc could rescue B cell differentiation in the absence of upstream signaling molecules known to be required for both myc expression and pre–B cell differentiation. In pre–B cells, myc genes are expressed, in part, by a pathway involving pre-BCR formation; the activation of Tec kinases, PLCγ, and PKC; and NF-κB translocation to the myc promoter (see Fig. 8; Grumont et al., 2002). First, we determined whether Myc could rescue B cell differentiation in the absence of pre-BCR formation by breeding Eμ-myc transgenic mice (Harris et al., 1988), which express c-myc exclusively in B lineage cells throughout development, to mice deficient in recombinase-activating gene 2 (RAG2; Shinkai et al., 1992), a gene required for the initiation of V(D)J recombination. RAG2−/− mice lack pre-BCR expression, resulting in a complete block in B cell development at the B220+CD43+CD25− pre–B cell stage (Fig. 3 A, Hardy fraction C). We find that c-myc Tg is sufficient to stimulate the differentiation of RAG2-deficient pro–B cells based on the acquisition of pre–B cell characteristics, including the down-regulation of CD43 and the up-regulation of CD25, IAb, CD22, and heat-stable antigen (HSA; Fig. 3, A–C). This translates to a rescue in the relative number of pre–B-like cells to a level equivalent to that of wild-type (Wt) mice based on an increase in the number and ratio of B220+CD43− cells to B220+CD43+ cells in BM from Eμ-myc/RAG2−/− as compared with RAG2−/− mice (unpublished data).
To further examine the proliferative capacity of Eμ-myc/RAG2−/− versus RAG2−/− B cell progenitors, we labeled BM cells with BrdU, which is incorporated into DNA during cell division, and with CFSE. The percentage of B220+ BrdU+ cells is higher in Eμ-myc/RAG2−/− compared with RAG2−/− mice (Fig. S2 A), and B cells from Eμ-myc/RAG2−/− mice undergo considerably more cell divisions than B cells from RAG2−/− mice in response to IL-7 stimulation (Fig. 3 E). In contrast, we do not see any difference in the number of apoptotic B220+ cells between Eμ-myc/RAG2−/− compared with RAG2−/− mice based on the acquisition of the apoptotic marker annexin V (Fig. S2 B).

Eμ-myc Tg rescues B cell maturation in mice deficient in Btk and Tec kinases

To further characterize the upstream signaling pathway involved in Myc-dependent differentiation, we determined whether Myc could rescue B cell differentiation in the absence of the Tec family PTKs, which mediate proliferation and differentiation downstream of the pre-BCR and BCR (see Fig. 8). We bred Eμ-myc mice to mice doubly deficient in expression of the Tec family PTKs Btk and Tec (Ellmeier et al., 2000). In humans, loss of function mutations in the btk gene lead to X-linked agammaglobulinemia, an immunological disease whereby Ca2+ signaling is impaired (Fluckiger et al., 1998; for review see Rawlings, 1999) and the generation of pre–B cells is blocked. In mice, pre–B cell development is only partially inhibited in btk−/− mice and proceeds normally in tec−/− mice but is essentially completely inhibited in btk/tec double-null mice. Thus, we asked whether Myc could rescue B cell development when Tec family signaling is ablated. As shown in Fig. 4 A, btk−/− tec−/− mice are deficient in their ability to generate pre–B cells based on a reduction in B220+CD43− B cell progenitors compared with Wt mice. However, when c-myc is expressed in btk−/− tec−/− B cell progenitors, both the percentage (Fig. 4 A) and total number (not depicted) of B220+CD43+ B cell progenitors, B220+CD22+, and B220+IAb+ pre–B cells and B220+IgM+ immature B cells are completely rescued. These results provide additional genetic evidence that Myc stimulates B cell development downstream of the pre-BCR and Tec family PTKs.

Figure 2. Deletion of c- and N-myc results in impaired B1 and B2 B cell development. (A) Splenic B cells from mice of the indicated genotypes (10–11 wk) were stained with antibodies to B220, IgM, and IgD. Relative numbers of total B cells (B220+ IgM−), follicular mature (IgMloIgDhi), T2 (IgMhiIgDhi), and T1/MZ (IgMhiIgDlo) B cells were determined as described in Materials and methods. Bars represent the mean ± SEM [error bars] obtained from six CD19cre− and four CD19cre+ mice. (B) Impaired B1 cell development in c-/N-myc-deficient mice. Peritoneal cells were stained with antibodies to B220, IgM, and CD5 and analyzed by flow cytometry. Numbers in dot plots indicate the percentage of CD5+IgM−, CD5+IgMhi (B1 B), and CD5+IgM+ (B2 B) populations. Data are representative of three separate experiments. (C) Decreased antibody production in myc-deficient mice. Mice were immunized with KLH-CFA, and the KLH-specific serum antibody concentrations were determined by ELSA. Bars represent the mean ± SEM of four Wt and seven CD19cre− c-mycfl/fl N-mycfl/fl mice. Serum dilutions are shown. dKO, double knockout.
Loss of Btk and Tec increases the tumor frequency in Eμ-myc transgenic mice

Because Tec kinases have been reported to both induce and suppress tumor formation (Hendriks and Kersseboom, 2005), we investigated how the transformation capacity of Eμ-myc Tg is altered by the loss of Btk and Tec. We find that the heterozygosity of btk and tec is sufficient to increase the tumor frequency in Eμ-myc mice, with the 50% tumor incidence being reduced from 100 d for Eμ-myc mice to 60 d for Eμ-myc/btk−/−tec−/+ (Fig. 4 B). The percent tumor-free incidence was decreased further as additional alleles of btk or tec were deleted. These results suggest that deregulated c-myc synergizes with the loss of Tec signaling during B cell lymphoma formation.

c-Myc-expressing B cells exhibit elevated Ca2+ levels and NFAT nuclear translocation

Ca2+ signaling is induced by the pre-BCR and is modulated by Btk (for review see Rawlings, 1999), and, in T lymphocytes (Aifantis et al., 2001) as well as in many other cell types, is required for the induction of differentiation. Because Myc rescues B cell differentiation in the absence of the pre-BCR and Btk, we investigated whether Myc can bypass requirements for normal Ca2+ signaling or whether Myc amplifies Ca2+ signaling in the absence of the pre-BCR or Btk. In B cells, the sustained Ca2+ flux after BCR cross-linking is thought to be caused by the concurrent activation of PI3K, which leads to the production of membrane-associated PI-3,4,5-P3 and to the recruitment and phosphorylation of Btk. Btk activation results in full and
sustained PLCγ activation, peak IP3 production, and maximal release of [Ca2+]i (see Fig. 8; for reviews see Rawlings, 1999; Hendriks and Middendorp, 2004).

To investigate whether Ca2+ signaling is affected during Myc-dependent differentiation, we labeled total BM cells from Eμ-myc Tg and Wt mice as well as Eμ-myc/RAG2−/− and RAG2−/− mice with the Ca2+-binding dye indo-1 (June and Rabinovitch, 1990) and fluorescent-conjugated antibodies against B cell markers and measured the ability of B cell progenitors to flux Ca2+ after stimulation with the Ca2+ ionophore ionomycin or anti-IgM. Surprisingly, we find that B cell progenitors from both Eμ-myc Tg and Eμ-myc/RAG2−/− mice exhibit elevated basal [Ca2+]i and prolonged duration of Ca2+ flux compared with Wt and RAG2−/− mice, respectively (Fig. 5 A and not depicted).Remarkably, Eμ-myc Tg also rescues peak and sustained [Ca2+]i in B cells (before and after anti-μ stimulation) in the absence of Btk and Tec kinases (Eμ-myc Tg/btk−/−/Tec−/−), which are the major mediators of sustained Ca2+ signaling downstream of the pre-BCR (Fig. 5 B). These results suggest that Myc amplifies [Ca2+]i signaling in B cells downstream of the pre-BCR and Tec kinases.

We next determined whether the elevated Ca2+ flux we observe in Myc-expressing B cells results in changes in known nuclear mediators of Ca2+ signaling. In normal T and B cells,
Ca²⁺ influx activates the phosphatase calcineurin (Cn), which dephosphorylates the NFAT family of transcription factors (NFATc1–4), allowing them to translocate from the cytoplasm to the nucleus and activate transcription of target genes (for reviews see Crabtree, 2001; Im and Rao, 2004). We examined the translocation of NFAT in purified splenic B cells from Eμ-myc Tg and normal Wt littermates both before and after BCR cross-linking.

**Figure 5.** Eμ-myc B cells exhibit elevated Ca²⁺ flux and enhanced NFATc nuclear translocation. (A) BM cells from Wt, RAG2−/−, or Eμ-myc/RAG2−/− mice were loaded with indo-1 dye and stained with αB220. Flow kinetics profiles are shown comparing the mean indo-1 ratio (violet/blue) of B220⁺ (top) and B220⁻ (bottom) cells as a function of time before and after stimulation with ionomycin. (B) Eμ-myc Tg rescues Ca²⁺ flux in Btk/Tec-null B cell progenitors. BM cells from mice of the indicated genotypes were loaded with indo-1 and stained with αB220 and αCD43. Kinetics profiles comparing the indo-1 fluorescence ratio of B220⁺ cells as a function of time before and after stimulation with anti-μ are shown. (C–F) B cells from Eμ-myc, Eμ-myc/RAG2−/−, and Eμ-myc/btk−/−tec−/− mice exhibit enhanced nuclear localization of NFATc. Cytoplasmic and nuclear extracts were prepared from splenic B cells stimulated with anti-μ for the indicated times (C, D, and F) or unstimulated purified B cell progenitors (E) and were size fractionated by SDS-PAGE. Western blot analysis was performed with αNFATc1 (C, E, and F) or αNFATc2 (D) or with loading controls β-actin (C–F), α-lamin (C–E), or α-Max (F). Adjacent panels depicted in D or E are from the same gel. White lines indicate that intervening lanes have been spliced out. R−/−, RAG2−/−; M/R−/−, Eμ-myc/RAG2−/−. NFAT isoforms are indicated by brackets or arrowheads.

Purified B cells from Wt littermates require 15 min of anti-IgM stimulation to achieve the maximal translocation of NFATc1 (Fig. 5 C and Fig. S5 C, available at http://www.jcb.org/cgi/content/full/jcb.200704173/DC1) and NFATc2 (Figs. 5 D and S5 D) to the nucleus. In contrast, B cells from Eμ-myc Tg mice have the majority of both NFATc1 and c2 in the nucleus, even before stimulation with anti-IgM. These effects are not the result of
increased NFAT expression in Eµ-myc B cells, as NFATc mRNA (Fig. S4 A) and protein levels (Fig. S5, A and B) are reduced or unchanged relative to Wt B cells. Myc expression also results in substantially increased nuclear NFATc1 translocation in unstimulated B cell progenitors from Eµ-myc/RAG2−/− compared with RAG2−/− mice (Figs. 5 E and S5 E) and reduces NFAT translocation in splenic B cells from Eµ-myc/btk−/−/ec− cells as compared with btk−/−/ec− mice (Figs. 5 F and S5 F). Transfection of Myc-null fibroblasts (HO.15 cells) with c-Myc (Shiio et al., 2002) results in greatly enhanced NFAT activity based on increased activation of an NFAT-luciferase reporter construct (Fig. S3 E), demonstrating that Myc also increases NFAT activity. These results suggest that the sustained increase in [Ca2+]i, signaling in Myc-expressing B cells has a major influence on key Ca2+-regulated nuclear events.

To assess whether the elevated Ca2+ flux in Myc-expressing B cell progenitors may contribute to the abilities of Myc to stimulate maturation and proliferation, we first determined how [Ca2+]i levels in these mice correlate with differentiation, proliferation, and survival status. We find that Ca2+ levels positively correlate with maturation and proliferation in Eµ-myc/RAG2−/− mice based on the down-regulation of CD43 on B220+ cells and dilution of CFSE. B cell progenitors with the most mature phenotype (B220+CD43−) and highest proliferative capacity have the highest levels of Ca2+ flux, whereas the cells with the least mature phenotype (B220−CD43hi-mid) and lowest proliferative capacity have the lowest levels of Ca2+ flux (Fig. S3, A–C). The observed increase in [Ca2+]i, is not the result of differences in cell cycle status because gated G0/G1 cells from Eµ-myc Tg have consistently elevated [Ca2+]i, relative to G0/G1 cells from normal littersmates (Fig. S3 D). These results suggest that there is a correlation between amplified Ca2+ signaling and the ability of Myc-expressing B cells to undergo differentiation and division.

We next examined whether normal Ca2+ signaling is required for maturation and division of Wt and Myc Tg pre-B cells. First, we determined whether impairing endogenous Myc expression results in impaired Ca2+ flux. Total BM cells from N-mycolg c-mycolg CD19cre− or CD19cre+ mice were stimulated in vitro with anti-µ for 2 h to induce myc expression followed by ionomycin stimulation to induce Ca2+ flux. As shown in Fig. 6 A, both pre-B/immature B and mature B cell populations from N-mycolg c-mycolg CD19cre+ mice exhibit reduced peak and sustained Ca2+ levels relative to CD19cre− mice. To determine whether a reduction of Ca2+ levels affects the ability of both Wt and Eµ-myc cells to proliferate and mature, we cultured total BM cells from Wt and Myc Tg mice in EGTA to limit the amount of available extracellular Ca2+ and assessed maturation and proliferative capacity after the addition of increasing doses of extracellular Ca2+. As shown in Fig. S4 (B and C), chelation of Ca2+ with EGTA results in the impairment in Ca2+ flux and proliferative capacity after ionomycin stimulation. EGTA also blocks the ability of Wt B cell progenitors to mature from the B220+CD43+ stage to the B220−CD43− stage (Fig. 6 B, first and second panels), which is rescued by the addition of extracellular Ca2+ (Fig. 6 B, third to fifth panels). B cell progenitors were unable to divide in low Ca2+ conditions based on limited CFSE dilution of live (TO-PRO-3) cells (Fig. 6 C, first and second panels), which is also rescued by the addition of extracellular Ca2+ (Fig. 6 C, third to fifth panels). Progenitors from Eµ-myc/RAG2−/− mice were also unable to mature (Fig. S4 D) or divide efficiently (not depicted) under low Ca2+ conditions.

As Eµ-myc B cells exhibit increased NFAT nuclear translocation, we also determined whether the Cn–NFAT pathway is required for the division of Wt and Eµ-myc B cells. Purified B cells from Wt and Eµ-myc mice were stimulated with anti-µ for 72 h in the presence or absence of cyclosporine A (CsA), a specific inhibitor of Cn. As shown in Fig. 6 D, Wt B cells undergo an average of four divisions (Fig. 6 D, left), and Eµ-myc B cells undergo an average of five divisions (Fig. 6 D, right) in response to anti-µ, whereas cell division is completely inhibited by CsA. Collectively, these results suggest that normal Ca2+ signaling is required for pre-BCR–mediated pre-B cell proliferation and maturation and for Myc to stimulate the differentiation of B cell progenitors.

**Myc regulates Ca2+ efflux**

The increase in [Ca2+]i that occurs in response to stimuli such as BCR ligation is transient, in part because Ca2+ is resequestered into the endoplasmic reticulum by the sarcoplasmic ER Ca2+ ATPase (SERCA) or is extruded from the cell by plasma membrane Ca2+-ATPase (PMCA) pumps. Because Myc amplifies [Ca2+]i in B cells in the absence of Btk and Tec (which regulate influx), we hypothesized that Myc could be altering the extent of Ca2+ influx. First, we measured the relative expression of PMCA1, PMCA4, and SERCA3, the respective pumps expressed in B lineage cells (Chen et al., 2004). Although we did not find consistent differences in the expression of PMCA1 and serca3 in mature B cells from Eµ-myc mice relative to Wt mice (unpublished data), levels of PMCA4 mRNA are significantly decreased in Eµ-myc mature B and Eµ-myc/RAG2−/− pro–B cells (Fig. 7 A), whereas PMCA4 mRNA is increased in sorted pro–B cells from N-mycolg c-mycolg CD19cre+ versus CD19cre− mice (Fig. S4 E). The decrease in PMCA4 expression correlates with a decrease in total PMCA protein (Fig. 7 B) and export of Ca2+ across the plasma membrane in purified B cells from Eµ-myc mice relative to Wt mice in response to anti-µ (Fig. 7 C), as measured by fluorometric analysis of extracellular media containing a membrane-impermeable version of the Ca2+-binding Indo-1 dye. These results suggest that Myc negatively regulates Ca2+ extrusion in B cells and are consistent with a recent report that Myc interacts with the human PMCA4b promoter, resulting in decreased PMCA4b expression (Zeller et al., 2006). Indeed, we also find using chromatin immunoprecipitation (ChIP) that Myc interacts within the homologous region in the mouse PMCA4 promoter (Fig. S4 F).

To determine whether reduced PMCA4b expression in Myc-expressing cells is important for Myc-induced proliferation, we infected BM cells from Wt or Eµ-myc mice with murine stem cell virus (MSCV) retroviruses containing internal ribosomal entry site (IRES)–GFP and human PMCA4b cDNA (MSCV-PMCA4b-GFP) or vector alone (MSCV-GFP). Analysis of the Phoenix retroviral packaging cell line indicates that MSCV-PMCA4b-GFP cells exhibit a substantially decreased baseline and peak [Ca2+]i, after ionomycin stimulation relative to GFP− cells,
whereas MSCV-GFP+ cells flux normally (Fig. 7 D). Infection of Wt and Eμ-myc BM with MSCV-PMCA4b-GFP virus results in increased hPMCA4b expression over MSCV-GFP–infected cells by real-time PCR (Fig. 7 E). MSCV-GFP–infected Wt and Eμ-myc pre–B cells (B220+CD43−) divide normally in response to IL-7 in vitro based on considerable dilution of the CFSE-like SNARF-1 dye. In contrast, B cell proliferation is inhibited in MSCV-PMCA4b-GFP–infected Wt and Eμ-myc B cells in a dose-dependent manner (Fig. 7 F and not depicted).

**Discussion**

**Myc stimulates B lymphocyte development**

The roles of Myc proteins in cell differentiation remains one of the most controversial issues in Myc biology. Early studies performed in cell lines and more recent studies in primary keratinocytes resulted in the prevailing view that Myc stimulates cell cycle entry while inhibiting differentiation (for review see Pelengaris et al., 2002). However, in primary chicken and murine B cells engineered to overexpress myc and in humans with Burkitt’s lymphoma, B lymphocytes mature to the Ig+ B cell stage relatively unperturbed despite the constitutive overexpression of myc throughout B cell development (Langdon et al., 1986; Thompson et al., 1987). These results suggest that B cells are able to mature relatively normally despite the presence of deregulated Myc.

In this study, we took a genetic approach to address the roles of Myc in the differentiation of mammalian cells using primary B cells. First, we found that conditional myc deletion significantly inhibits B cell development at the pro–B to pre–B cell transition (Fig. 1 B), which is the stage at which the combined
signaling from the pre-BCR and IL-7 normally induce c- and N-myc expression. Second, to distinguish the ability of Myc to drive the proliferation of postdifferentiated cells from its ability to initiate a differentiation program, we asked whether Myc could rescue differentiation on two genetic backgrounds (RAG2−/− and btk−/−/tec−/−) whereby B cell development is blocked just before the stages at which c- and N-myc are normally expressed. We found that the Eμ-myc Tg efficiently stimulated the differentiation and expansion of pre-B-like cells from pro-B cells in the absence of pre-BCR formation (in RAG2−/− mice). Additionally,
we found that Myc can rescue B cell development to the IgM+ immature B cell stage in the context of a btk−/−/tec−/− background, whereby B cell development is nearly completely blocked at the large pre–B cell stage. These results collectively provide genetic evidence that Myc acts downstream of the pre-BCR and Btk/Tec to stimulate pre–B cell development and are consistent with previous studies suggesting that the pre-BCR is required for myc induction and pre–B cell development (Zimmerman and Alt, 1990; for review see Hendriks and Middendorp, 2004).

The notion that Myc may initiate differentiation was first proposed by Gandalillas and Watt (1997) in keratinocytes in vitro. Recent studies have suggested roles for Myc in inducing the differentiation of hematopoietic and epidermal stem cells, in part by regulating the expression of adhesion molecules, thus releasing them from a differentiation-inhibiting niche (Murphy et al., 2005). In this study, we show that constitutive Myc can act in a cell-autonomous manner to rescue differentiation in lineage-committed progenitor cells. These results collectively suggest that the phenotypic consequences of Myc during development may depend on the differentiation status of the cell: Myc expression in stem cells and lineage-committed progenitors results in differentiation, whereas Myc expression in mature postmitotic cells results in reentry into the cell cycle and inhibition of terminal differentiation.

Deregulated Myc synergizes with the loss of Tec kinase function during lymphoma formation

Although btk-null or btk/tec double-null mice do not spontaneously develop pre–B cell tumors, the absence of both btk and SLP-65 significantly enhances the incidence of pre–B cell leukemia as compared with SLP-65−/− mice (Hendriks and Kersseboom, 2005). Although the exact mechanism of tumor suppression by Tec family PTKs is not known, the loss of Btk and/or SLP-65 in B cells results in sustained IL-7 receptor (IL-7R) expression and an increased proliferative and/or survival response to IL-7. Here, we show that loss of Btk/Tec significantly accelerates B cell tumor formation in Eμ-myc mice (Fig. 4 B). In addition, we find that Eμ-myc Tg substantially increases the proliferative potential of B cell progenitors in response to IL-7, whereas decreasing myc expression inhibits IL-7–mediated proliferation. Thus, sustained IL-7R expression in tec/btk-null mice, in cooperation with constitutive Myc activity as occurs in Burkitt’s lymphoma, substantially increases the pool of dividing cells that is capable of acquiring epigenetic alterations that promote tumorigenesis. We conclude that Tec PTKs act as tumor suppressors that attenuate deregulated c-myc and that IL-7 synergizes with Myc to increase the pool of dividing B cell progenitors. The ability of Myc to drive differentiation to the IgM-positive stage in Btk−/−/Tec−/− mice while having accelerated transformation further suggests that it is not the inhibition of maturation itself but rather the degree of growth factor responsiveness that determines tumor susceptibility. Consistent with the latter notion, loss of Jak3 kinase, an essential IL-7 signaling molecule, reduces the transforming potential of the Eμ-myc Tg (Dillon and Schlissel, 2002).

Myc regulates Ca2+ signaling and NFAT translocation during B cell development

Because Ca2+ signaling is important for virtually all aspects of embryogenesis (for review see Webb and Miller, 2003) and pre-BCR and Btk stimulate Ca2+ signaling pathways, we were prompted to investigate how Ca2+ signals were affected during Myc-dependent B cell development. Surprisingly, we found that both basal [Ca2+]i levels and the duration of Ca2+ flux are elevated in B cells from Eμ-myc Tg and Eμ-myc/RAG2−/− mice. Furthermore, an increase in the relative level of [Ca2+]i, in Eμ-myc/RAG2−/− Tg mice positively correlates with increased proliferation and differentiation and is required for Myc to stimulate B cell development and proliferation. The increase in [Ca2+]i, level may occur by down-regulation of the Ca2+ efflux pump PMCA4b because Myc interacts with the PMCA4b promoter, and both PMCA4b expression and Ca2+ efflux are decreased in Eμ-myc B cells. Moreover, enforced PMCA4b expression inhibits Myc-induced cell proliferation. These results are consistent with a study in developing T lymphocytes in the thymus, which depend on Ca2+ signaling during the maturation and expansion of CD4 CD8− pre–T cells after rearrangement of the T cell receptor β chain and formation of the pre–T cell receptor (Aifantis et al., 2001). In addition, gene-targeted mutations in other genes involved in Ca2+ signaling, including the Src family PTKs Lyn/Fyn/Btk, Syk, SLP-65, LAT (linker for activation of T cells), Btk/Tec, the p85 subunit of PI3K (Fruman et al., 1999), and PLCγ1/2 (Wen et al., 2004), also result in impaired B cell development at the pre–B cell stage. Altogether, these results strongly support a role for Myc and Ca2+ signaling in the maturation of pre–B cells.

A study in primary B lymphocytes indicates that the amplitude and duration of Ca2+ signals have profound influences on the type of transcriptional response (Dolmetsch et al., 1997). For example, NF-κB and JNK are selectively activated by a large transient [Ca2+]i, rise, whereas NFAT is activated by lower but sustained [Ca2+]i levels. We also find that B cells from Eμ-myc Tg mice, which exhibit elevated, sustained [Ca2+]i levels after antigenic stimulation (Fig. 5), also exhibit increased NFAT translocation even in the absence of upstream signals from the pre-BCR and BCR. In addition, B cell nuclear fractions from Myc Tg mice appear to contain mostly the short isoform of NFATc1 (NFATc1A; Fig. 5), which, in contrast to the other NFATc1 isoforms (NFATc1B and c1C), does not promote apoptosis in lymphocytes (Chuvpilo et al., 2002). These results suggest that the elevated [Ca2+]i, in Myc-expressing B cells has profound influences on transcriptional and biological responses as well. Indeed, we find that anti-IgM–induced B cell proliferation of both Wt and Myc Tg B cells is completely abrogated by treatment of cells with CsA, and a recent study indicates that the B cell–specific deletion of Cn results in defects in B cell proliferation and function (Winslow et al., 2006).

Implications of Myc-regulated Ca2+ signaling

One common feature of many types of cancers is their reduced dependence on external growth factors, and Myc-induced tumors share this property. Indeed, Myc activates at least three genetic programs that are normally growth factor dependent, including cyclin E/Cdk2 kinase, E2F-dependent transcription, and protein
synthesis pathways (for review see Eisenman, 2001). How can one transcription factor with limited target gene specificity induce so many biochemical and biological changes that are normally dependent on multiple signaling pathways? Our results suggest that although Myc directly stimulates the expression of many E box–containing genes, Myc also increases sustained \([Ca^{2+}]_{i}\) and enhances NFAT translocation (Fig. 8). This allows for the concurrent activation of \(Ca^{2+}\)-dependent target genes even in the apparent absence of upstream mediators of \(Ca^{2+}\) signaling, as might occur under conditions of poor growth factor availability.

Figure 8. **Model of Myc and \(Ca^{2+}\) signaling events downstream of the pre-BCR/BCR.** Engagement of the pre-BCR/BCR results in the recruitment of Src family kinases (Blk, Lyn, and Fyn), phosphorylation of the Igα–Igβ complex, and formation of a \(Ca^{2+}\) signaling module containing SLP-65, PI3K, Blk/Tec, and PLCγ. Full activation of PLCγ by Blk results in IP3-mediated \(Ca^{2+}\) release from the ER, resulting in activation of the Cn–NFAT signaling pathway. PKCβ activation by \(Ca^{2+}\) and DAG leads to the nuclear translocation of NF-κB and the induction of \(myc\) expression. Our results implicate Myc in amplifying \([Ca^{2+}]_{i}\), by decreasing PMCA4-mediated \(Ca^{2+}\) efflux, thereby promoting optimal NFAT-dependent proliferation, differentiation, and immunogenic B cell responses. Induction of low level \(Ca^{2+}\) signals or Myc signaling alone results in a tolerogenic and/or apoptotic response, whereas Myc activation together with sustained \(Ca^{2+}/NFAT\) signals or constitutive Myc signaling alone promotes B cell activation/differentiation or tumorigenesis.
or limited extracellular Ca\(^{2+}\). The synergy between Myc and [Ca\(^{2+}\)]\(_i\) to stimulate B cell development and proliferation is strikingly similar to the signaling requirements underlying immunogenic versus tolerogenic responses to antigen in B lymphocytes. Although activation of Myc alone or a low level Ca\(^{2+}\) signal alone provokes apoptosis or anergy (tolerance; Glynne et al., 2000), Myc expression in conjunction with a sustained Ca\(^{2+}\)/NFAT signal results in a mitogenic response and immunity. Here, we find that Myc amplifies [Ca\(^{2+}\)], thus tuning levels into the range required for optimal NFAT activation and translocation. The unique function of Myc to amplify Ca\(^{2+}\) signaling may help explain why transformed cells are relatively resistant to low growth factor and Ca\(^{2+}\) conditions (Whitfield, 1992) despite known requirements for Ca\(^{2+}\) signaling during G1→S transition.

**Materials and methods**

**Mice**

CS7BL/6 J 6.5 mcg transgenic mice were obtained from Jackson ImmunoResearch Laboratories and were genotyped by PCR according to instructions from the supplier. To generate mice carrying conditionally inactivated c- and N-myc genes, mice carrying a floxed c-myc allele (c-myc\(^{opa}\)) de Alboran et al., 2001) were bred to N-myc\(^{opa}\) mice (Knopfli et al., 2002) expressing the cre recombinase. Tg under the control of the CD19 promotor (Ricket et al., 1997). C57BL/6 E-Tg mice were obtained from Jackson Immunoresearch Laboratories and were genotyped by PCR according to instructions from the manufacturer.

**Cell culture**

For cell division experiments, cells were stained with CFSE according to the manufacturer’s instructions (Invitrogen). CFSE-labeled cells were cultured in complete RPMI + 10% FBS (HyClone) in the presence or absence of 10 ng/ml murine recombinant IL7 (R&D Systems) or 5 μg/ml anti-μ (Fab\(^{2}\) fragment; Thermo Fisher Scientific) for the indicated times at 37°C and 5% CO\(_2\). In some experiments, E7A1 was added at a final concentration of 0.5, 0.75, or 1 μM to IL7-driven cultures in the presence or absence of 0.2, 0.4, or 0.6 mM Ca\(^{2+}\), and cyclosporine was added at a final concentration of 100 ng/ml to anti-μ-driven cultures. Thereafter, cells were stained with the indicated antibodies for analysis by FACS or harvested for measurement of [Ca\(^{2+}\)]\(_i\). For simultaneous analyses of Ca\(^{2+}\) flux and DNA content, fluo-4 Ca\(^{2+}\) binding dye (Invitrogen) was added at a final concentration of 1 μM in place of indo-1 acetoxymethyl ester (Sigma-Aldrich) or 1 μg/ml ionomycin (EMD) as indicated. For pre-BCR cross-linking of c- and N-myc-deficient or Wt BM, indo-1–loaded cells were first preincubated with 25 μg/ml anti-μ for 2–3 h at 37°C.

**Measurement of extracellular Ca\(^{2+}\) and DNA content**

For measurement of [Ca\(^{2+}\)]\(_i\), cells were washed and resuspended in 0.5 ml HBSS containing 3% FBS at 10^−10^ cells/ml for harvested BM cultures or 5 × 10^5–8 × 10^5 cells/ml for freshly isolated BM). EGTA-treated cell cultures were resuspended in Ca\(^{2+}\)-free HBSS/3% FBS. Indo-1–acetoxymethyl ester (penta-acetoxymethyl ester; Sigma-Aldrich) was added at a final concentration of 10–20 μM, and incubation was performed for 30 min at 37°C. The Indo-1 fluorescence ratio (400:530 nm) of the cells was acquired as a function of time using a flow cytometer (BDLSR 1; Becton Dickinson). For each experiment, collection of a 30 s baseline measurement was followed by stimulation with 0.25 μg/ml anti-μ or 1 μg/ml ionomycin (EMD) as indicated. For pre-BCR cross-linking of c- and N-myc-deficient or Wt BM, indo-1–loaded cells were first preincubated with 25 μg/ml anti-μ for 2–3 h at 37°C.

**Reporter gene assays**

For NFAT-luciferase assays, HO.15 (Myc null) or HO.15 + c-Myc fibroblasts were plated in 24-well tissue culture dishes at 90% confluence and, 24 h later, were transfected in triplicate with pNFAT-luciferase plasmid or pCI-SISK NK negative control plasmid containing the luciferase reporter gene without any cis-acting elements (Strategene) and the pRL Renilla luciferase control reporter vector (Promega) using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Treatment with 60 ng/ml PMA + 1 μg/ml ionomycin or PMA/ionomycin + 100 ng/ml CsA was performed 18 h after transfection. After 6 h, luciferase assays were performed with a luminometer (Monolight 1500; Analytical Luminescence Laboratories) and the Dual Luciferase Reporter assay system (Promega). Firefly luciferase values for each transfection were normalized to Renilla luciferase activity (pRL), and data were expressed as relative luciferase activity versus that obtained with the pCI-SISK negative control plasmid. Mean values of a representative experiment of three performed are displayed ± SEM.

**B cell purification**

Single-cell suspensions of total splenocytes and BM cells were isolated as previously described (Irntini et al., 1997). B lymphocytes were enriched by

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**Used in this study include:**

B220-PE-Cy-5.5 (eBioscience), FITC anti-mouse CD21, biotin anti-mouse CD22, biotin anti-mouse IA\(^\text{a}\) (BD Biosciences), FITC-annexin V (Invitrogen), IgM-PE-Cy7 (SouthernBiotechno), and IgD-FITC (BD Biosciences). Cytometry was performed on FACSscan and LSR flow cytometers (BD Biosciences). Data were analyzed using CellQuest (Becton Dickinson) and FlowJo (version 6.3.2; Tree Star, Inc.) softwares. To determine relative numbers of B lineage cells in each population (Fig. 1 B), percentages from flow cytometry were multiplied by the total BM cellularity to obtain absolute numbers of pro-B cells (B220 \(\text{CD}^{+}\)43\(^{-}\)), pre-B cells (B220 \(\text{CD}^{+}\)43\(^{+}\)), immature B cells (B220 \(\text{Mig}^{+}\)), and mature B cells (B220 \(\text{Mig}^{+}\)). To determine relative numbers of peripheral B2 B cells in each population (Fig. 2 A), percentages from flow cytometry were multiplied by the total spleen cellularity to obtain absolute numbers of total B cells (B220 \(\text{Mig}^{+}\)), follicular mature (\(\text{Mig}^{+}\)G0\(^{+}\)), T2 (\(\text{Mig}^{+}\)G0\(^{-}\)), and 11/MAZ (\(\text{Mig}^{+}\)G0\(^{-}\)) B cells.
negative selection on magnetic microbeads coupled to anti-mouse CD43 (Milenyi Biotec) or using the mouse B cell Negative Isolation kit (Invitrogen) according to protocols provided by the manufacturers. The recovered splenic B cells were rested in RPMI 1640 medium before stimulation for biochemical analyses or were washed into efflux buffer for Ca2+ efflux assays. B lineage cells were enriched from total BM by positive selection on magnetic beads coupled to CD45R/B220 (Invitrogen) or CD19 (Millenyi Biotec) according to the manufacturers’ instructions or by cell sorting on a FACSaria (BD Biosciences).

Western blot analysis
Purified splenic B cells were stimulated with 25 μg/ml anti-μ, [Fab’]2 fragment in serum-free RPMI medium. At the indicated time points, the cells were washed twice in ice-cold PBS and resuspended in hypotonic buffer containing proteinase inhibitors. Cytoplasmic and nuclear extracts were prepared by the method of Dolmetsch et al. (1997). SDS-PAGE and Western blotting were performed using standard techniques. Anti-NFATc1, anti-NFATc2, anti-β-actin, anti-Max, and donkey anti-goat Ig-HRP were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-mouse Ig HRP secondary antibody was purchased from Invitrogen. Goat anti-rabbit Ig HRP secondary antibody was purchased from Sigma Aldrich. Anti-PMCA monoclonal antibody (clone 5F10) was purchased from Millipore. Anti–lamin A/C antibody was purchased from Cell Signaling Technologies. Anti-α-tubulin monoclonal antibody was purchased from Sigma-Aldrich.

ChIP
ChIP splenic B cells from Myc Tg mice were cross-linked with 1% formaldehyde for 10 min at 37°C. ChIP was performed with the ChIP assay kit (Millipore) according to the manufacturer’s instructions using anti-c-Myc (N-262; Santa Cruz Biotechnology, Inc.) or control rabbit IgG (IgG; Santa Cruz Biotechnology, Inc.) antibodies. After reversal of cross-links, DNA was precipitated and detected by qPCR with pairs of primers (underlined) (Millipore) according to the manufacturer’s instructions using anti–c-Myc antibodies. Deletion of c-myc alleles has been described previously (de Alboran et al., 2001; Knoepfl er et al., 2002). Deletion of c-myc was quantified by real-time PCR measuring the fold enrichment relative to background detected with rabbit IgG and normalized to β-actin. Primers used to amplify hypoxanthine-guanine phosphoribosyl transferase (hprt) were as follows: hp rt forward (5'-TTGTGATACAGGCCAGACTTGGT-3') and hp rt reverse (5'-GAGGCTAGCTGCTTAAAGC-3'). Primers used to amplify NFATc1 and NFATc2 have been described previously (Hayashi et al., 2003). Quantitative deletion analysis of c-myc alleles was performed using genomic DNA from FACS-sorted CD19+ or cre− c-mycfl/fl and pre-B cells and primers specific for the floxed c-myc allele (de Alboran et al., 2001) using 45S measurement to normalize. Experiments were performed using a real-time PCR system sequence detector (model 7300; Applied Biosystems) and a PCR system (Mx4000; Stratagene).

KLH immunization
Mice were immunized with KLH protein (Calbiochem) emulsified in complete Freund’s adjuvant (CFA; 1:1 vol/vol mixture of 1 mg/ml of sterile protein solution-CFA) by subcutaneous injection at the base of the tail. Two injection sites were administered with 50 μl of the mixture. Mice were killed after 7 d. KLH-specific antibody production was measured with a KLH-coated ELISA system. For IgM, IgG1, and IgG2a measurements, sera were diluted 1:200, 1:135, and 1:135, respectively.

Statistical analysis
One-tailed t test was used for all analyses except the Kaplan-Meier analyses, in which we used Prism software (version 4; GraphPad) to generate two-tailed p values.

Image acquisition and manipulation
Developed films were scanned with a positive (version 8.0; Adobe), and adjustments of contrast and brightness were performed with Photoshop software. Scanned images were imported into Canvas (version 9.0.2; ACD Systems of America) for figure preparation. In reference to Fig. 5, the nuclear α-lamin control Western blots shown in Fig. 5 C and E and the β-actin control Western blot shown in Fig. 5 F were obtained by probing a separate gel. For evaluation of c- and N-myc deletion by densitometry (Fig. 5 I), band intensities representing the ratio of deleted to floxed alleles (with background subtracted) are shown below each lane and were quantified by densitometry on an imaging system (Alpha Innotech 3400; Alpha Innotech).

Online supplemental material
Fig. S1 shows the specific deletion of c- and N-myc in B lineage cells. Fig. S2 shows that the Eμ-myc Tg suppresses proliferation but not apoptosis of RAG2−/− B cell progenitors or IgH exclusion in Wt B cell progenitors. Fig. S3 shows that the elevated Ca2+ flux of Eμ-myc B cell progenitors correlates with increased maturation and proliferation and increased NFAT activity. Fig. S4 shows that chelation of Ca2+ impairs Ca2+ flux, maturation, and proliferation of Wt and Eμ-myc B cell progenitors in vitro, that Myc interacts with the mouse PMCA4 promoter in Eμ-myc B cells, and real-time PCR analyses of NPAC message from purified splenic B cells and PMCA4 message from c- and N-myc-deleted pro-B cells. Fig. S5 shows total levels of NFAT protein in whole cell lysates from Wt and Eμ-myc B cells and fractionation controls for the separation of cytoplasmic and nuclear fractions depicted in Fig. 5 (C–F). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704173/DC1.

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