Cell, Vol. 81, 223–231, April 21, 1995, Copyright © 1995 by Cell Press

Three Distinct IL-2 Signaling Pathways Mediated by *bcl-2*, c-*myc*, and *lck* Cooperate in Hematopoietic Cell Proliferation

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Summary

Two interleukin-2 receptor–dependent signaling pathways have thus far been identified: the c-*fos*/c-*jun* induction pathway mediated by *src* family protein-tyrosine kinases and the c-*myc* induction pathway. Here, we provide evidence for the existence of a third, rapamycin-sensitive pathway, which results in the induction of another proto-oncogene, *bcl-2*. In the hematopoietic cell line BAF-B03, the expression of any two of *lck*F505 (an active form of p56^{lck}), Bcl-2, or c-Myc is sufficient to promote transit of the cell cycle, regardless of the activation state of the third pathway. We also provide evidence that epidermal growth factor receptor signaling may act through the same pathway that involves p56^{lck}. These studies demonstrate a novel approach to dissecting signaling pathways regulating cellular proliferation.

Introduction

The functional interleukin-2 receptor (IL-2R) consists of three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγ chains, none of which contain an intrinsic protein-tyrosine kinase (PTK) domain that is the hallmark of other growth factor receptors (reviewed by Waldmann, 1989; Ullrich and Schlessinger, 1990; Taniguchi and Minami, 1993). We have shown previously that expression of the human IL-2Rβ cDNA in BAF-B03, an interleukin-3 (IL-3)-dependent murine hematopoietic cell line that expresses IL-2Rα and IL-2Rγ but not IL-2Rβ, allows the cells to proliferate in response to IL-2. Expression studies with mutant human IL-2Rβ cDNAs have revealed that a mutant (S mutant) lacking the membrane-proximal cytoplasmic region, termed the serine-rich region (S region), cannot transmit proliferative signals (Hatakeyama et al., 1989). More recently, evidence has also been provided demonstrating the critical role of the IL-2Rγ cytoplasmic region in IL-2 signaling (Kawahara et al., 1994; Nakamura et al., 1994; Nelson et al., 1994), pointing to the importance of cooperation between the IL-2Rβ and IL-2Rγ cytoplasmic domains in activating downstream signaling pathways. Despite extensive structural and functional studies on the IL-2R components, information is still limited with respect to the nature and function of the signaling pathways that they activate and their target genes.

Although the IL-2R components themselves lack PTK domains, IL-2 stimulation evokes tyrosine phosphorylation of intracellular proteins. Notably, the IL-2Rβ chain interacts both physically and functionally with members of the *src* family of nonreceptor PTKs; p56^{lck} is activated by IL-2 in peripheral blood lymphocytes (PBLs) (Hatakeyama et al., 1991; Horak et al., 1991), and other *src* family members, p59^{fyn} and p53/56^{lyn}, are activated in an analogous manner in BAF-B03-derived cells (Torigoe et al., 1992; Kobayashi et al., 1993). The physical interaction between p56^{lck} and IL-2Rβ is mediated by the PTK domain and the "acidic region" (A region), respectively (Hatakeyama et al., 1991). Moreover, the activation of *src* family PTKs by IL-2 requires the S region of IL-2Rβ, and this activation correlates both with p21^{ras} activation (Satoh et al., 1992) and with induction of the nuclear proto-oncogenes c-*fos* and c-*jun* (Shibuya et al., 1992; Minami et al., 1993). Recently, it was found that the S region of IL-2Rβ is necessary for the binding of two distinct PTKs, Jak1 of the Janus family PTKs (Miyazaki et al., 1994; Witthuhn et al., 1994) and Syk PTK of the Syk/ZAP-70 family PTKs (Minami et al., 1995) and that the carboxy-terminal region of IL-2Rγ is necessary for the association of Jak3 (Johnston et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). However, the role of these PTKs in the activation of *src* family PTKs is still unknown. A mutant form of the IL-2Rβ chain lacking the A region (A mutant) is deficient in activating the p56^{lck} PTK pathway (Minami et al., 1993), but it is still capable of bringing about IL-2-induced proliferation when expressed in BAF-B03 cells (Hatakeyama et al., 1989). Hence, this raises the issue of whether the p56^{lck} PTK pathway is involved in any way in IL-2-induced cell proliferation.

Expression studies with mutant IL-2Rβ and epidermal growth factor receptor (EGFR) cDNAs in BAF-B03 cells revealed the presence of another IL-2R-linked pathway

leading to the induction of the nuclear proto-oncogene c-*myc* (Shibuya et al., 1992). This pathway is activated by the wild-type and the A mutant but not by the S mutant IL-2Rβ chains. EGFR stimulation results in the activation of the c-*fos*/c-*jun* pathway but not the c-*myc* pathway, and the c-*myc* gene must be ectopically expressed for the EGF-stimulated cells to progress beyond S phase of the cell cycle (Shibuya et al., 1992). Hence, c-*myc* is a target of the IL-2R-mediated signal that is required for the progression of the hematopoietic cell cycle. Constitutive expression of c-*myc*, however, does not cause IL-2 (or IL-3)-independent growth of BAF-B03 cells (Shibuya et al., 1992). Taken together, these observations indicate that an additional, as yet unidentified target gene(s) is required for cell proliferation. This gene is activated by the IL-2R, does not respond to p56^{lck}, and must cooperate with c-*myc* (see Figure 1).

In the present study, we have reexamined the genes involved in BAF-B03 cell proliferation and have adduced evidence that *bcl-2* is another target gene that mediates IL-2-induced cell proliferation. *bcl-2* was originally identified as an oncogene that is amplified in follicular lymphoma (Tsujimoto and Croce, 1986), and it has been extensively studied in the context of the inhibition of apoptosis (reviewed by Korsmeyer, 1992). Recently, in addition to *bcl-2*, a *bcl-2*-related gene, *bcl-x*$_L$, has also been demonstrated

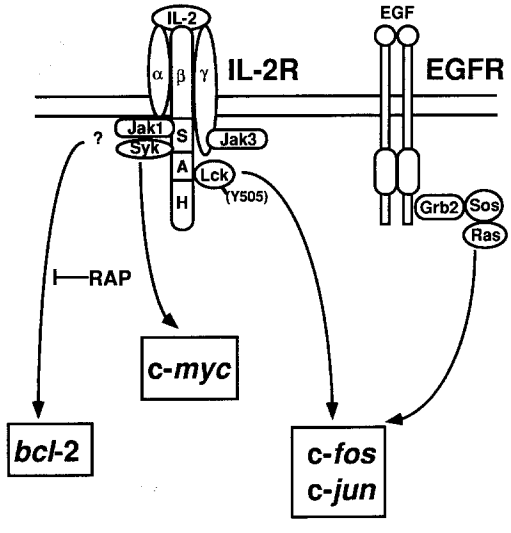

Figure 1. A Model for Three Distinct Signaling Pathways Diverging from IL-2R

This figure summarizes our conclusion that IL-2R stimulation is linked to three distinct signaling pathways: the p56^{lck} activation pathway (which leads to c-*fos*/c-*jun* induction), the c-*myc* induction pathway, and the RAP-sensitive, *bcl-2* induction pathway. The physical interaction between p56^{lck} and IL-2Rβ requires the A region of the IL-2Rβ chain. Induction of both the c-*myc* and *bcl-2* genes is mediated by the S region of the IL-2Rβ chain. The S region of IL-2Rβ is necessary for binding of two distinct PTKs, Jak1 of the Janus family PTKs and Syk PTK of the Syk/ZAP-70 family PTKs. The activation of Syk PTK is linked to the induction of the c-*myc* gene. The H region of IL-2Rβ is essential for Stat5 activation. The carboxy-terminal region of IL-2Rγ is necessary for the association with Jak3. The role of Jak1 and Jak3 PTKs in the induction of these proto-oncogenes remains unknown (see text for details).

to inhibit apoptosis (Boise et al., 1993). In contrast, another *bcl-2*-related gene, *bax*, has been shown to accelerate apoptosis (Oltvai et al., 1993). The induction of *bcl-2*, *bcl-x*$_L$, or *bax* by IL-2 also requires the S region of the IL-2Rβ chain; however, only *bcl-2* induction is inhibited by rapamycin (RAP) (Figure 1). We demonstrate that c-*myc* and *bcl-2* can cooperate in stimulating cell proliferation, in that coexpression of these two genes leads IL-2 (IL-3)-independent cell proliferation. Moreover, we observed that an activated form of p56^{lck} carrying a phenylalanine substitution at position 505 (p56^{lck}F505) can cooperate with either c-Myc or Bcl-2 in the induction of cell proliferation, providing strong evidence supporting a role for p56^{lck} in transmitting mitogenic signals. We also examined cooperation of either of p56^{lck}F505, c-Myc, or Bcl-2 with EGFR stimulation.

Results

Induction of *bcl-2* Gene by IL-2R

It has been shown that many hematopoietic cell lines undergo apoptosis in the absence of cytokine stimulation and that the product of the *bcl-2* gene prolongs cell survival and blocks the apoptosis (reviewed by Korsmeyer, 1992; Collins and Rivas, 1993; Vaux, 1993). It has been demonstrated also that IL-2 stimulation induces *bcl-2* gene expression (Deng and Podack, 1993; Otani et al., 1993); however, a role for Bcl-2 in IL-2 signaling is not well established.

To examine if and how the *bcl-2* gene is involved in IL-2-induced cell proliferation, we first studied *bcl-2* mRNA induction in BAF-B03 cells expressing wild-type or mutant IL-2Rβ chains (i.e., F7, A15, and S25 cells). The F7 cells express the wild-type IL-2Rβ chain, the A15 cells express a mutant IL-2Rβ chain lacking the A region (amino acid residues 313–382), and the S25 cells express an IL-2Rβ chain lacking the S region (amino acid residues 267–322). It has been previously shown that A15 cells can respond to IL-2, albeit less well than F7 cells, whereas S25 cells remain unresponsive (Hatakeyama et al., 1989). When F7 cells were stimulated with IL-2, *bcl-2* mRNA induction could be seen after 30 min and reached its peak level after 12 hr (Figure 2). The induction of the *bcl-2* gene was also observed in A15 cells after IL-2 stimulation, albeit at a lower level than in F7 cells. IL-2 stimulation of S25 cells, however, did not result in *bcl-2* mRNA expression (Figure 2). These results indicate that the S region of IL-2Rβ, which is essential for growth signal transmission, is also necessary for *bcl-2* gene induction. The S region was also found to be essential for the induction of the *bcl-x*$_L$ and *bax* genes (Figure 2; data not shown).

bcl-2 Gene Induction Is Sensitive to Rapamycin

To dissect the IL-2 signaling pathways further, we next examined the effects of two immunosuppressive drugs, FK506 and RAP, on IL-2-induced proto-oncogene expression. These drugs are known to bind to the same intracellular target, but the latter selectively affects the IL-2-dependent T cell proliferation (Sigal and Dumont, 1992). As shown in Figure 3A, neither of the drugs showed any effect on the induction of c-*fos*, c-*jun*, or c-*myc* genes by IL-2.

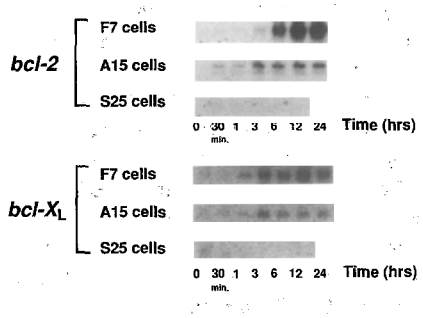

Figure 2. Expression of the *bcl-2* and *bcl-x*$_L$ Genes in BAF-B03-Derived Transformants Stimulated with IL-2

Northern blot analysis of mRNA expression in F7, A15, and S25 cells. Total RNA was extracted from cells that had been synchronized (by factor starvation for 15 hr) and then stimulated with IL-2 (2 nM) for various periods. Northern blot analysis was carried out as described in Experimental Procedures, and autoradiographs were exposed for 3 days. Expression of Bcl-2 protein was confirmed by Western blot analysis.

On the other hand, RAP but not FK506 was found to selectively retard *bcl-2* gene induction in a profound manner; when F7 cells were stimulated with IL-2 in the presence of RAP, the expression of *bcl-2* mRNA was delayed for 6–9 hr. However, RAP showed no effect on the induction of *bcl-x*$_L$ and *bax* mRNAs (Figure 3B; data not shown). This suggests that *bcl-2* gene induction may be one of the targets of RAP. IL-2-induced cell cycle progression as well as the induction of some cell cycle stage–specific genes was also affected by RAP (data not shown). There is thus a close correlation between the IL-2-induced expression of the *bcl-2* gene and progression through the cell cycle.

bcl-2 Cooperates with EGF Signaling to Elicit Cell Proliferation in a c-*myc*-Independent Manner

It has been shown previously that, in BAF-B03-derived BER2 cells, c-*myc* plays a critical role in the progression of the cell cycle up to the G2/M phase. BER2 cells have been engineered to express the human EGFR cDNA, and EGF stimulation causes the uptake of [^{3}H]thymidine, but does not elicit cell cycle progression, unless c-*myc* is also expressed ectopically (Shibuya et al., 1992). In view of the fact that neither the c-*myc* gene nor the *bcl-2* gene is induced by EGF stimulation in BER2 cells (Shibuya et al., 1992; Figure 4A[a]), we tested the ability of *bcl-2* to drive cell cycle progression in BER2 cells. The human *bcl-2* cDNA was cloned into the expression vector pSVT, and the resulting construct pSVBT was cotransfected with the hygromycin (*hgr*) resistance gene into BER2 cells. Three clones expressing the transfected *bcl-2* cDNA, BB3, BB8, and BB13, were obtained. These clones did not proliferate in a growth factor–independent fashion but could be induced to proliferate in response to EGF stimulation (Figure 4B). The BB cells continued to proliferate in response to EGF stimulation for at least 1 month. All the *hgr*$^+$ clones that were cotransfected with the control vector pSVT failed to proliferate in response to EGF (data not shown).

RNA blotting analysis with the mouse IL-3 cDNA probe did not show any IL-3 mRNA expression in BB3, BB8, and BB13 cells cultured in the EGF-containing medium.

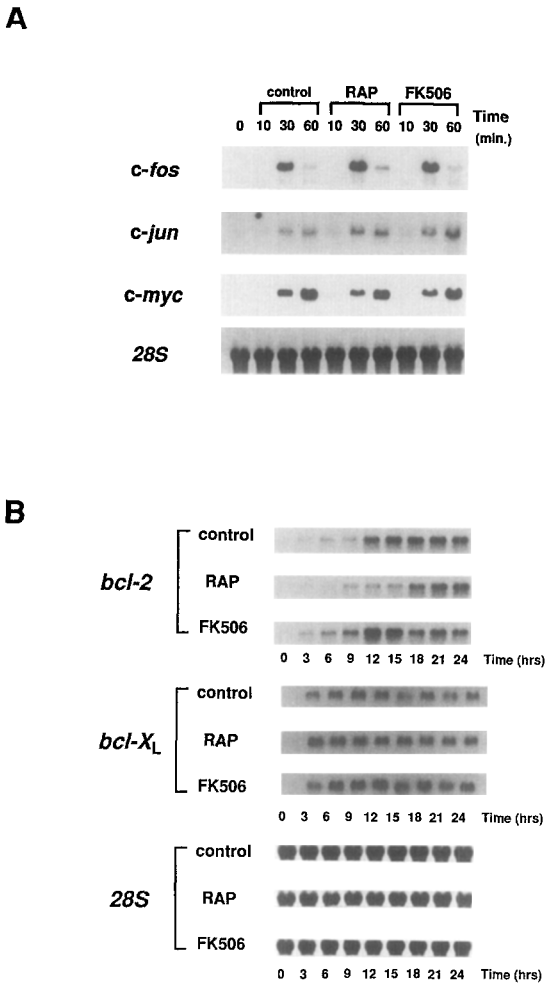

Figure 3. Effects of RAP on the Expression of Proto-Oncogenes and *bcl-x*$_L$ Gene Induced by IL-2

(A) RAP has no effect on the expression of c-*fos*, c-*jun*, and c-*myc* proto-oncogenes in F7 cells following IL-2 stimulation. Synchronized F7 cells (2×10^7) were preincubated with culture medium or culture medium containing either RAP (10 ng/ml, Wyeth-Ayerst Laboratories) or FK506 (10 ng/ml, Fujisawa Chemical) for 30 min and stimulated with IL-2 (2 nM) for various periods. Northern blot analysis was carried out as described in Figure 2. Autoradiographic exposure times were 3 hr (c-*myc*), 3 days (c-*fos*), and 6 days (c-*jun*). Membranes were stained with methylene blue and stained 28S ribosomal RNA was indicated. (B) RAP affects the *bcl-2* gene but not *bcl-x*$_L$ gene induction in F7 cells by IL-2 stimulation. Synchronized F7 cells (2×10^7) were preincubated with culture medium or culture medium containing the respective drugs RAP (10 ng/ml) or FK506 (10 ng/ml) for 30 min and stimulated with IL-2 (2 nM) for various periods. Autoradiographic exposure times were 1 day (*bcl-2*) and 3 days (*bcl-x*$_L$). Membranes were stained with methylene blue and stained 28S ribosomal RNA was indicated.

In addition, supernatant from EGF-stimulated BB3, BB8, and BB13 cells did not support the proliferation of BER2 or F7 cells (data not shown), indicating that cell proliferation is directly mediated by the combination of EGF signaling and *bcl-2* expression rather than the induction of growth stimulatory cytokines. EGF stimulation of BER2 cells did not induce the N-*myc*, *bcl-x*$_L$, or *bax* genes (Figure 4A[a]; data not shown). Importantly, EGF stimulation of BB3 cells did not induce c-*myc* or N-*myc* expression (Figure 4A[b]),

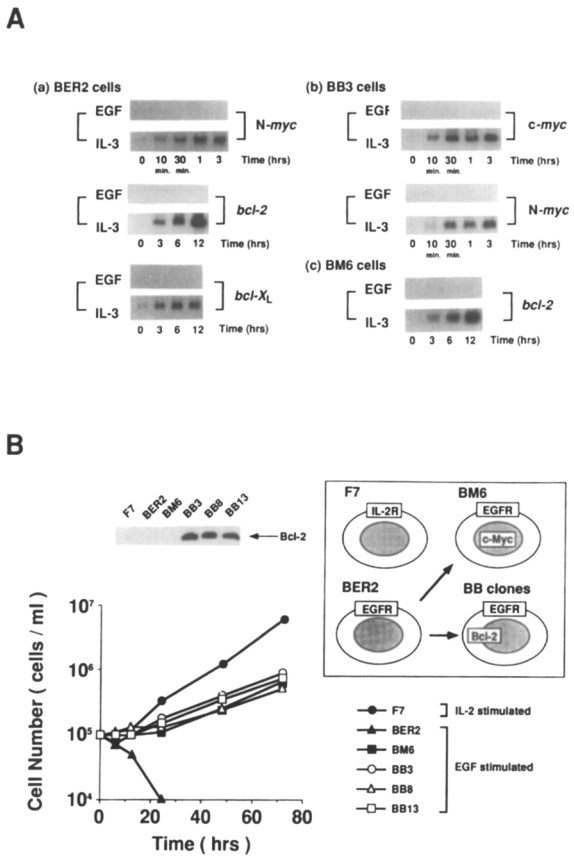

Figure 4. Expression of the Exogenous *bcl-2* Gene in BER2 cells Has No Effect on *myc* Genes Induction but Confers Responsiveness to EGF for Cell Proliferation

(A) (a) Expression of N-*myc*, *bcl-2*, and *bcl-x*$_L$ genes in factor–stimulated BER2 cells. After synchronized BER2 cells were stimulated with either EGF or IL-3 for various periods, total RNA extracted from them was subjected to Northern blot analysis.

(b) Expression of c-*myc* and N-*myc* genes in factor-stimulated BB3 cells. After synchronized BB3 cells were stimulated with either EGF or IL-3 for various periods, total RNA extracted from them was subjected to Northern blot analysis.

(c) Expression of *bcl-2* gene in factor-stimulated BM6 cells. After synchronized BM6 cells were stimulated with either EGF or IL-3 for various periods, total RNA extracted from them was subjected to Northern blot analysis.

(B) Proliferation profiles for the factor-stimulated F7, BER2, BM6, BB3, BB8, and BB13 cells. Expression of the human Bcl-2 was assessed by Western blot analysis and is indicated by the arrow (left inset). Synchronized cells were plated at 1 × 10^5 cells/ml in the presence of IL-2 (F7 cells) or EGF (others). The number of viable cells was counted at various times after factor stimulation and represented on a logarithmic scale. The IL-3R is not shown in the right inset panel.

suggesting that cell proliferation indeed occurs in the absence of *myc* gene expression. Uptake of [^{3}H]thymidine following EGF stimulation was almost the same between the parental BER2 cells and the BB3 clone (data not shown), suggesting that the expression of the *bcl-2* gene in these clones did not alter the levels of DNA synthesis. Thus, progression of the cell cycle in BAF cells can be brought about by the cooperation between EGF signaling and either c-*myc* or *bcl-2* expression.

We also examined whether expression of the endogenous *bcl-2* gene is affected by c-*myc* expression. The BER2-derived BM6 cells constitutively express human c-Myc from a transfected expression plasmid containing the human c-*myc* gene, and these cells continued to proliferate in response to EGF stimulation for at least 1 month (Shibuya et al., 1992; data not shown). As shown in Figure 4A(c), *bcl-2* mRNA induction was not detectable in BM6 cells after EGF stimulation. These observations indicate that the c-*myc* and *bcl-2* genes do not affect the expression of each other and, therefore, that Bcl-2 and c-Myc independently cooperate with EGFR stimulation to promote the cell cycle progression.

Cooperation of c-Myc and Bcl-2 Enables BAF-B03 Cells to Proliferate Independent of Cytokines

To clarify further the nature of the cooperation between Bcl-2 and c-Myc in cell proliferation, we generated cell transformants that constitutively express both the human c-*myc* and *bcl-2* genes (BMB). The puromycin (*puro*) resistance gene and either an expression vector for human *bcl-2* gene (pSVBT) or control vector (pSVT) were cotransfected into a cell line, BM6. Whereas the parental BM6 cells were unable to proliferate unless they were stimulated with either EGF or IL-3, the *puro*$^+$ clones BMB7, BMB17, and BMB23, which express the transfected *bcl-2* gene, could proliferate in a growth factor–independent fashion (Figure 5A). BMB cells continued to proliferate in the absence of either IL-3 or EGF for at least 1 month at a rate approximately 70% of that of the IL-3-stimulated parental BER2 cells (data not shown). Evidence for the cooperation of c-*myc* and *bcl-2* in lymphoid tumorigenesis has been provided previously (Vaux et al., 1988; Strasser et al., 1990).

Cooperation of p56^{lck} PTK with c-Myc or Bcl-2 for Cell Proliferation

To address the issue of whether the *src* family PTKs contribute to IL-2-induced cell proliferation, we next examined whether p56^{lck} could cooperate with either of the two known target genes of IL-2 signaling, c-*myc* and *bcl-2*. This seemed especially important to test since the induction of c-*myc* and *bcl-2* genes occurs in A15 cells that express a mutant IL-2Rβ deficient for the *src* family PTK activation and since the activation of the *src* family PTKs, like the activation of an artificially introduced EGFR, leads to the induction of the c-*fos*/c-*jun* genes, but not the c-*myc* and *bcl-2* genes (Shibuya et al., 1992; Minami et al., 1993; Figures 2 and 4A[a]; T. M., unpublished data).

Mutation of the conserved carboxy-terminal tyrosine residue (505) of p56^{lck} to phenylalanine (p56^{lck}F505) gives rise to a p56^{lck} variant with much increased PTK activity (Marth et al., 1988). An expression vector for p56^{lck}F505, termed pdKCR-lckF505, was constructed and cotransfected with the *hgr* resistance gene into BAF-B03-derived BER2 cells expressing the human EGFR (Shibuya et al., 1992). Several transformants expressing the active form of p56^{lck} (p56^{lck}F505) were obtained, and these clones were termed BF cells. These BF clones were unable to proliferate upon EGF stimulation, indicating the lack (or insufficiency) of cooperation between EGFR signaling and p56^{lck}

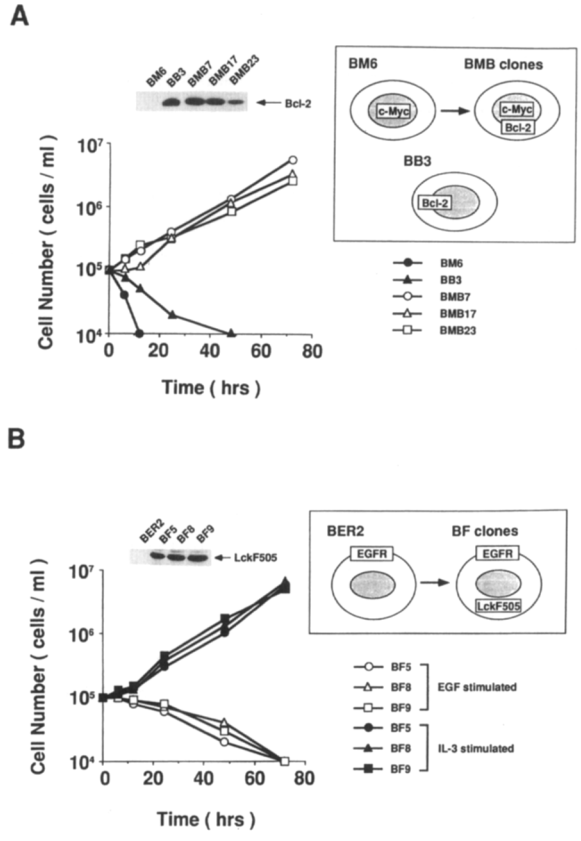

Figure 5. Proliferation Profiles for BMB and BF Cells

(A) Factor independency of the BMB7, BMB17, and BMB23 cells for cell growth. Expression of Bcl-2 was assessed by Western blot analysis and is indicated by the arrow (left inset). Synchronized BM6, BB3, and BMB cells were plated at 1 × 10^5 cells/ml in the absence of IL-2, IL-3, and EGF. Coexpression of the c-*myc* and *bcl-2* genes in BAF-B03 cells also resulted in IL-3-independent proliferation. These cells did not express detectable levels of IL-3 mRNA by RNA blotting analysis, and the culture supernatant was unable to support proliferation of BER2 or F7 cells, thus indicating that the cell proliferation observed is not mediated by endogenously produced growth factors.

(B) Insufficiency of cooperation between EGF signaling and p56^{lck}F505 in BF cells. Expression of p56^{lck}F505 was assessed by Western blot analysis and is indicated by the arrow (left inset). Synchronized BF cells were plated at 1 × 10^5 cells/ml in the presence of IL-3 or EGF.

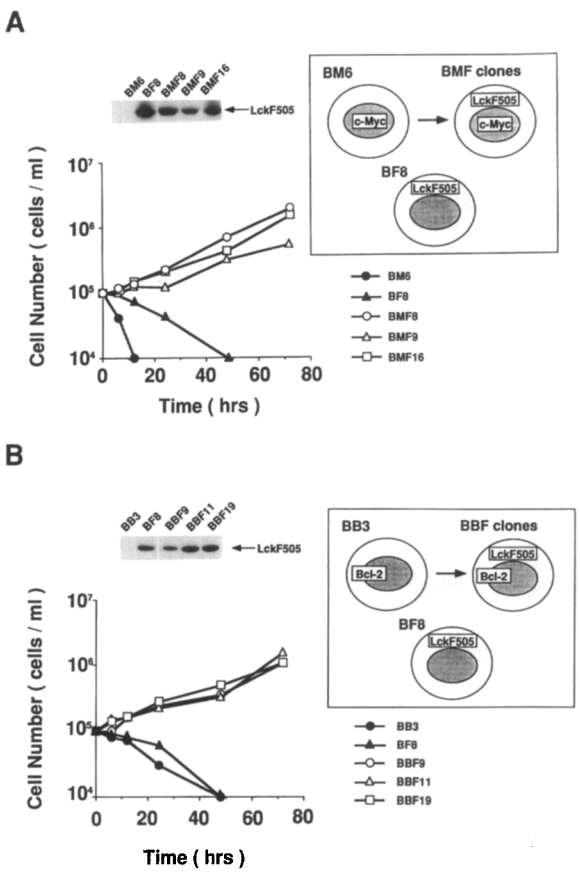

Figure 6. Factor Independency of the BMF and BBF Cells for Cell Growth

Proliferation profiles for the BM6, BF8, BMF8, BMF9, and BMF16 cells (A) and the BB3, BF8, BBF9, BBF11, and BBF19 cells (B) in the absence of growth factor. Expression of p56^{lck}F505 was assessed by Western blot analysis and is indicated by the arrows (left inset). Synchronized cells were plated at 1 × 10^5 cells/ml in the absence of IL-2, IL-3, and EGF. The density of viable cells was counted at various times after plating and represented on a logarithmic scale. The IL-3R and EGFR are not shown in the right inset panel.

(Figure 5B). Next, pdKCR-lckF505 was similarly cotransfected with the *puro* resistance gene into the BAF-B03-derived BM6 and BB3 cells that constitutively express the *c-myc* and *bcl-2* genes, respectively. Transformants expressing p56^{lck}F505 were obtained, and these were termed BMF and BBF cells, respectively. Unlike BF cells, both BMF and BBF cells could proliferate in a growth factor–independent manner (Figures 6A and 6B). Supernatants from these cells showed no detectable growth factor activity (data not shown). These observations indicate that p56^{lck} activation per se is insufficient to promote cell proliferation but that it can cooperate with either c-Myc or Bcl-2, in a manner analogous to EGFR stimulation. Taken together, these observations suggest that the IL-2R is linked to at least three pathways leading to the activation of target genes critical for proliferative signal transmission (i.e., the *src* family PTK-linked c-*fos*/c-*jun* induction pathway, the c-*myc* induction pathway, and the RAP-sensitive, *bcl-2* induction pathway).

Suppression of Apoptosis by p56^{lck}

It has been demonstrated that c-Myc induces apoptosis when expressed in the absence of serum or growth factors (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992), whereas Bcl-2 suppresses it (Bissonnette et al., 1992). When BER2, BM6, or BB cells were starved of IL-3 for 15 hr and the number of viable cells subsequently was counted at various intervals, accelerated cell death, accompanied by DNA fragmentation (data not shown), was observed in BM6 cells constitutively expressing the human c-*myc* gene, compared with the parental BER2 cells. In contrast, BB cells expressing the human *bcl-2* gene could survive for a prolonged period relative to BER2 cells (Figure 7A). Interestingly, BF cell lines, which express an active form of p56^{lck}, could survive in the absence of growth factors for a prolonged period relative to the parental BER2 cells (Figure 7B), and DNA fragmentation was also sup-

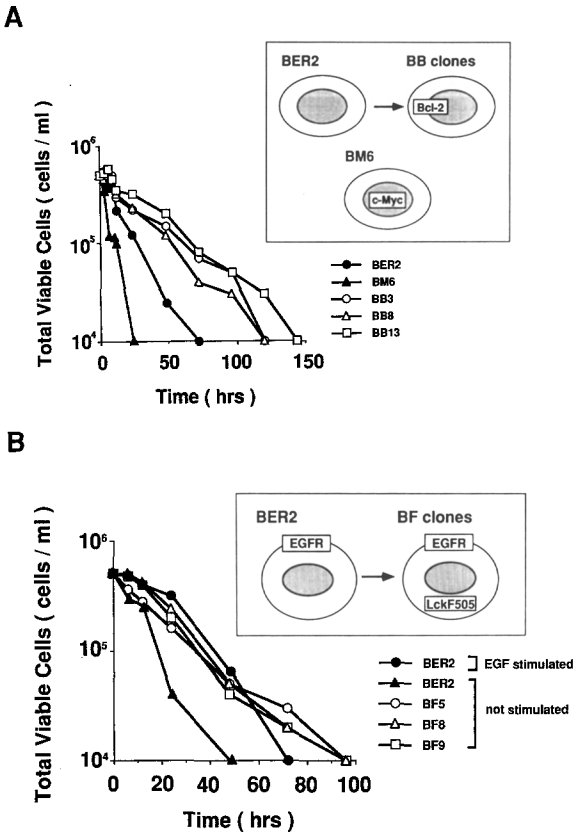

Figure 7. Regulation of Apoptosis in BAF-B03 Derived Cells by c-*myc*, *bcl-2*, or *lck*F505 Genes Expressed Exogenously

(A) Cell viability of BER2, BM6, BB3, BB8, and BB13 cells following factor deprivation. Synchronized cells were plated at 5 × 10^5 cells/ml in the absence of IL-2, IL-3, and EGF. The density of viable cells was counted at various times after plating and represented on a logarithmic scale.

(B) Cell viability of BF5, BF8, and BF9 cells following factor deprivation and of the parental BER2 cells with or without EGF stimulation. Synchronized cells were plated at 5 × 10^5 cells/ml with or without EGF stimulation. The number of viable cells was counted at various times after plating. The IL-3R and EGFR are not shown in the right inset panel.

pressed (data not shown). In all BF clones, constitutive expression of an active form of $p56^{lck}$ did not affect *bcl-2* mRNA expression in the absence of growth factors (data not shown). In addition, EGFR stimulation of the parental BER2 cells also resulted in suppression of cell death (Figure 7B). These observations suggest that, like Bcl-2, $p56^{lck}$ activation, as well as EGFR stimulation, results in the suppression of apoptosis.

Discussion

Many cytokine/cytokine receptor systems have been extensively studied in the context of the regulation of cell proliferation, particularly in hematopoietic lineages. It has been shown that IL-2 induces tyrosine phosphorylation of a number of intracellular proteins and that the IL-2R is coupled both physically and functionally with multiple nonreceptor-type PTKs (see Introduction). Despite the identification of such PTKs as downstream mediators of IL-2 signal transmission, the nature of their target genes and the mechanisms whereby they promote cell cycle progression are not fully understood.

bcl-2 as a Critical Target in IL-2 Signaling

In the present study, we have provided evidence that, in addition to c-*fos*/c-*jun* and c-*myc* genes, another proto-oncogene (*bcl-2*) is critical for IL-2 signaling. Analysis of BAF-B03-derived clones expressing IL-2Rβ mutants has revealed that the induction of both the c-*myc* and *bcl-2* genes is mediated by the S region of the IL-2Rβ chain. It is interesting to note, however, that RAP inhibits *bcl-2* gene induction but not c-*myc* gene induction. Hence, our observations suggest the presence of a RAP-sensitive intracellular IL-2 signaling pathway leading to *bcl-2* gene induction and indicate that the induction pathways for *bcl-2* and c-*myc* must diverge at a point beyond that which is mediated by the S region of the IL-2Rβ chain. Recently, a target protein of the RAP–FKBP12 complex, termed FRAP/RAFT1, has been identified that appears to be a mammalian homolog of the yeast TOR proteins (Brown et al., 1994; Sabatini et al., 1994). In view of the fact that both TOR and FRAP/RAFT1 proteins show homology to lipid kinases, one might speculate a link between such kinases and the *bcl-2* induction.

As a result of the finding that IL-2-induced *bcl-2* gene expression is selectively affected by RAP, we established F7-derived transformants that constitutively expressed the *bcl-2* gene and examined the sensitivity of these cells to RAP. These clones proliferated better than the parental F7 cells in the presence of RAP, but remained partially sensitive to RAP (T. M., unpublished data). This may reflect the fact that other biochemical pathways are also RAP sensitive. For example, it is known that RAP inhibits the activation of $p70^{S6K}$ (Chung et al., 1992; Kuo et al., 1992), although at present the relationship between $p70^{S6K}$ activation and *bcl-2* induction by IL-2 is unclear.

Our present findings provide evidence for the importance of the *bcl-2* proto-oncogene in the regulation of the hematopoietic cell cycle. *bcl-2* gene induction is selectively inhibited by RAP, and this correlates with the inhibition of cell cycle progression. The cell cycle progresses when Bcl-2 expression is accompanied by the expression of c-Myc or $p56^{lck}$F505, or by EGFR signaling in BAF-B03-derived cells. Thus far, the function of Bcl-2 has been extensively studied in the context of suppression of apoptosis (reviewed by Korsmeyer, 1992; Schwartz and Osborne, 1993; Collins and Rivas, 1993; Vaux, 1993). In this context, our results may be interpreted to mean that EGFR stimulation or constitutive expression of c-Myc or $p56^{lck}$F505 leads to simultaneous proliferative and apoptotic stimuli and that Bcl-2 suppresses the apoptotic stimulus, resulting in cell proliferation (see Harrington et al., 1994). On the other hand, our results suggest another possibility: Bcl-2 may also play a role in promoting hematopoietic cell proliferation independent of its role in the inhibition of apoptosis. While these two possibilities cannot be rigorously distinguished at present, the following observations argue for the latter possibility: first, although c-Myc

can provide an apoptotic stimulus in BAF cells, both EGFR stimulation and p56^{lck}F505 expression, each of which also cooperates with Bcl-2, in fact suppress apoptosis (Figure 7B); second, in BB cells, which express Bcl-2 constitutively, cyclin D3 mRNA, but not the D1 and D2 mRNAs, remains elevated after cytokine deprivation, whereas cyclin D3 expression declines in the parental BER2 cells. In fact, constitutive expression of cyclin D3 along with either c-Myc or p56^{lck}F505 expression allows the cytokine-independent proliferation of BAF cells. In contrast, cyclin D3 and Bcl-2 do not cooperate in this manner (Z.-J. L. and T. M., unpublished data). Although further work will be required to elucidate the precise mechanism by which Bcl-2 affects the cell cycle, the results presented here nevertheless provide strong evidence that *bcl-2* induction by IL-2 (or IL-3) is critical for cell cycle progression.

The Role of p56^{lck} in IL-2-Induced Cell Proliferation

Our results with the active form of p56^{lck} (p56^{lck}F505) also suggest a role for this PTK in the transmission of the IL-2-induced mitogenic signal(s). Previously, we had shown that the IL-2Rβ mutant lacking the acidic region (A mutant) fails to associate with and activate the p56^{lck} PTK but still induces cell proliferation (Hatakeyama et al., 1989; Minami et al., 1993). In view of the results presented here, it is most likely that cells expressing the A mutant proliferate in response to IL-2 stimulation via the induction of c-*myc* and *bcl-2* in the absence of p56^{lck} activation. Consistent with this notion is the observation that expression of these two genes in combination can render BAF-B03-derived cells cytokine independent (Figure 5A).

Furthermore, coexpression of p56^{lck}F505 with either c-Myc or Bcl-2 also promotes cell cycle progression in the absence of cytokines. p56^{lck}F505 expression, however, cannot cooperate with EGF stimulation to promote cell proliferation (Figure 5B). In addition, p56^{lck}F505, as well as EGFR stimulation, appears to suppress apoptosis upon IL-3 deprivation in BAF-B03-derived cells (Figure 7B). We infer that the p56^{lck} PTK elicits a signal similar or identical to that of the EGFR (Figure 1). In fact, it has been shown previously that c-*fos*, c-*jun* and their family members can be activated by a p56^{lck}-linked pathway or by EGFR signaling in BAF-B03 cells (Shibuya et al., 1992).

Cooperation of the Three Signaling Pathways

Our results indicate the existence of at least three distinct signaling pathways linked to the IL-2R: the p56^{lck} pathway that leads to c-*fos*/c-*jun* induction, the *bcl-2* induction pathway, and the c-*myc* induction pathway (Figure 1). Importantly, none of these pathways affects the activation of the other: c-*myc* gene expression does not affect the induction of c-*fos*/c-*jun* or *bcl-2*, *bcl-2* gene expression does not affect c-*fos*/c-*jun* or c-*myc* expression, and p56^{lck} PTK activation does not induce the *bcl-2* or c-*myc* genes (Figures 2 and 4A; T. M., unpublished data).

Expression studies show that a combination of any two of the three pathways is sufficient to promote the growth of BAF-B03 cells in the absence of cytokines (Figures 5A, 6A, and 6B). We infer that these three pathways cooperate with each other to ensure a full-scale signal transmission by IL-2. Consistent with this are the observations that F7 cells expressing wild-type IL-2Rβ respond to IL-2 better than A15 cells that express the IL-2Rβ A mutant (Shibuya et al., 1992) and that BMB cells that are able to proliferate in the absence of cytokines via the constitutive expression of c-*myc* and *bcl-2* can also respond to EGF as indicated by an apparent further increase in the rate of proliferation (approximately 1.5-fold; T. M., unpublished data).

The induction pathways of both the c-*myc* and *bcl-2* genes are linked to the S region of the IL-2Rβ chain. Recently, it was found that this region of IL-2Rβ is necessary for the binding of Jak1 (Miyazaki et al., 1994; Witthuhn et al., 1994) and Syk PTK (Minami et al., 1995) and that the carboxy-terminal region of IL-2Rγ is necessary for association with Jak3 (Johnston et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). It was also shown that the activation of Syk PTK results in the induction of the c-*myc* gene (Minami et al., 1995; Figure 1). Thus, it is likely that Jak1, Jak3, and Syk are involved in the induction of the above proto-oncogenes by IL-2. More recently, we have found that IL-2 activates Stat5 in F7 cells. This Stat5 activation (presumably by the IL-2R-associated Jak PTKs) requires the membrane distal H region, yet this region is dispensable for IL-2-induced cell proliferation (H. Fujii and T. T., unpublished data; Figure 1; Hatakeyama et al., 1989). Therefore, it is unlikely that Stat5 activation is involved in the induction of these proto-oncogenes.

Although in our experimental system activation of two out of three pathways was required for cell proliferation, it is possible that activation of all three pathways may be required for the proliferation of certain cells, e.g., peripheral T cells, depending on the magnitude of induction of these target genes. In this regard, it is important to note that our inferences have certain limitations, since they are primarily based on the results of constitutive gene expression assays, whereas the induction of these genes is normally of a transient nature. The question of how the signaling mechanisms operating in BAF-B03 cells relate to other cells, such as peripheral T cells, also remains to be addressed. Nevertheless, in view of the findings that IL-2-induced activation of *src* family PTKs (the Syk PTK, Jak1 and Jak3 PTKs) is observed and that all of the target genes (c-*fos*/c-*jun*, c-*myc*, and *bcl-2*) are also induced by IL-2, in both normal lymphocytes and BAF-B03 derived cells, we think that BAF-B03 cells retain similar, if not identical, mechanisms of cell proliferation. The fact that RAP, which selectively inhibits the *bcl-2* gene induction pathway, inhibits the growth of T cells (Sigal and Dumont, 1992) and BAF-B03 cells (data not shown) is also consistent with this view. At the same time, our results do not exclude the possibility of additional IL-2R-linked signaling pathways.

Experimental Procedures

Cells and Cell Culture

BAF-B03, a subclone of BAF3 cell line, is a bone marrow–derived murine IL-3-dependent pro-B cell line (Palacios and Steinmetz, 1985; Collins et al., 1992). F7 is a BAF-B03-derived stable transformant clone expressing the wild-type human IL-2Rβ chain. A15 and S25 are also BAF-B03-derived stable transformant clones expressing the human

IL-2Rβ mutant lacking the cytoplasmic "acidic" region and the "serine-rich" region, respectively (Hatakeyama et al., 1989). BER2 is a BAF-B03-derived stable transformant clone that expresses the human EGFR. BM6 is a BER2-derived stable transformant clone expressing the human c-*myc* gene ectopically (Shibuya et al., 1992). BB cells were established by transfecting the human *bcl-2* expression plasmid pSVBT (containing the cDNA for human *bcl-2* gene; Tsujimoto and Croce, 1986) into BER2 cells. BF cells were obtained by transfecting the mouse mutant p56^{lck} expression plasmid pdKCRlckF505 into BER2 cells. BMB and BMF cells were obtained by transfecting the plasmids pSVBT and pdKCRlckF505 into BM6 cells, respectively. BBF cells were obtained by transfecting the plasmids pdKCRlckF505 into BB3 cells. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 10% (v/v) conditioned medium from the WEHI-3B cell line (10% WEHI-conditioned medium) as a source of IL-3.

For the analyses of gene expression, cell growth, and cell viability, cells were stimulated with either WEHI-conditioned medium (at a final concentration of 10% [v/v]) or recombinant human EGF (at a final concentration of 10 ng/ml), after cells were synchronized in G1 phase by depriving them of cytokines for 15 hr (the cell viability after cytokine deprivation was more than 90%).

DNA Transfection

Plasmid DNAs were transfected into the cells by an electroporation procedure as described previously (Doi et al., 1989). Selection was initiated 24 hr after transfection, using 1 mg/ml hygromycin for BB or BF cells and 7.5 μg/ml puromycin for BMB, BMF, or BBF cells. Drug-resistant colonies were picked up and subsequently cloned by limiting dilution as described previously (Miyazaki et al., 1991).

Preparation of Probe DNA

The probe DNAs for c-*myc*, c-*fos*, and c-*jun* were prepared as described previously (Shibuya et al., 1992), and the probe for human and mouse *bcl-2* were prepared as follows: human *bcl-2*, 1.0 kb XhoI fragment from the plasmid pSVBT; mouse *bcl-2*, 2.5 kb HindIII fragment from the plasmid pmbcl3.3 (Negrini et al., 1987). The probe for *bax* was a PCR fragment (nucleotides 215–569) amplified from a murine spleen cDNA library. The probe for *bcl-x*$_L$ was the 0.8 kb EcoRI fragment from the plasmid pBS-bcl-x$_L$ (Boise et al., 1993).

RNA Extraction and Northern Blot Analysis

Total cellular RNA from each cell was prepared as described previously (Hatakeyama et al., 1989). For RNA blot analysis, 10 μg of total RNA was loaded on each slot (Shibuya et al., 1992).

Western Blot Analysis

Cells (2×10^6) were harvested and subjected to the analysis as described previously (Minami et al., 1993). The primary antibodies are rabbit anti-p56^{lck} antiserum 195.7 or anti-human Bcl-2 monoclonal antibody /100.

Cell Growth Assay and Viability Assay

For the cell growth assay, factor-starved cells were cultured at a density of 1×10^5 cells/ml in RPMI-1640 supplemented with 10% FCS containing either human EGF (10 ng/ml) or human IL-2 (2 nM) or WEHI-conditioned medium (10% [v/v]). Culture media were changed every 2 days (Miyazaki et al., 1991). For the cell viability assay, factor-starved cells were cultured in PRMI-1640 supplemented with 10% FCS without EGF or cytokines. In both assays, viable cell numbers were determined by trypan blue exclusion assay.

Acknowledgments

We thank Drs. S. Korsmeyer, G. Thompson, and T. Kondo for providing us with the *bax*, *bcl-x*$_L$ and N-*myc* cDNAs, Dr. A. P. Baker and Fujisawa Chemical for RAP and FK506. We also thank Dr. A. Kukula for critical reading of the manuscript and Drs. M. Lamphier and Y. Eguchi for valuable comments. This work was supported in part by a Grant in Aid for Special Project Research, Cancer Bioscience, from the Ministry of Education, Science, and Culture of Japan. The first two authors contributed equally to this work.

Received October 7, 1994; revised February 16, 1995.

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Note Added in Proof

The work on Stat5 that is cited as unpublished data by H. Fujii and T. T. in the Discussion is now in press: Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J. N., Minami, Y., Miyazaki, T., and Taniguchi, T. (1995). Activation of Stat5 by IL-2 requires a carboxyl-terminal region of the IL-2 receptor β chain but is not essential for the proliferative signal transmission. Proc. Natl. Acad. Sci. USA, in press.