Direct regulation of \textit{BCL-2} by FLI-1 is involved in the survival of \textit{FLI-1}-transformed erythroblasts

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Rearrangement of the \textit{FLI-1} locus with ensuing overexpression of \textit{FLI-1} is an early event in Friend murine leukemia virus-induced disease. When overexpressed in primary erythroblasts, FLI-1 blocks erythropoietin (Epo)-induced terminal differentiation and inhibits apoptosis normally induced in response to Epo withdrawal. We show here that the survival-inducing property of FLI-1 is associated with increased transcription of \textit{BCL-2}. We further show that FLI-1 binds \textit{BCL-2} promoter sequences in transformed erythroblasts, and \textit{in vitro} studies identify specific FLI-1-binding sites essential for the transactivation of the \textit{BCL-2} promoter by FLI-1. Analysis of FLI-1 mutants showed a correlation between the ability of FLI-1 to transactivate \textit{BCL-2} promoter sequences and their ability to inhibit apoptosis in the absence of Epo. Moreover, inhibitor studies confirmed the essential role of \textit{BCL-2} for \textit{FLI-1}-transformed erythroblast survival. Finally, enforced expression of \textit{BCL-2} was sufficient to promote survival and terminal differentiation of erythroblasts in the absence of Epo. These results show that \textit{BCL-2} is an \textit{in vivo} target of FLI-1 in \textit{FLI-1}-transformed erythroblasts and that its deregulated expression is instrumental in the survival of these cells. 

\textbf{Keywords:} \textit{BCL-2}/erythroblast survival/FLI-1/Friend erythroleukemia

Introduction

The \textit{ETS} gene family encodes transcriptional regulators that are essential for a variety of cellular processes and for the response of cells to developmental and extracellular cues (for a review see Ghysdael and Bourex, 1997). The \textit{ETS} domain of these proteins binds DNA response elements (EBS) in the regulatory regions of \textit{ETS} protein target genes. Other domains are involved in either activation or repression of transcription, with specificity being conferred both at the level of DNA binding and through interactions of \textit{ETS} proteins with unrelated transcriptional regulators.

\textit{ETS} proteins play a central role in a variety of oncogenic processes. Transformation by signal transduction oncogenes such as \textit{RAS} is critically dependent upon \textit{ETS} proteins (Wasylyk \textit{et al.}, 1994). Moreover, several solid tumors and leukemias are linked specifically to mutation or abnormal expression of \textit{ETS} proteins. For example, the hallmark of Ewing sarcoma is the fusion of the 5' half of the \textit{EWS} gene to the 3' part of one of several \textit{ETS} genes, most often \textit{FLI-1} or \textit{ERG}, as the result of specific chromosomal translocations (Delattre \textit{et al.}, 1992). The chimeric proteins encoded by the \textit{EWS–FLI-1/EWS–ERG} fusion oncogenes are aberrant transcriptional regulators of EBS-driven transcription (Ballay \textit{et al.}, 1994). They interact with several components of the basal transcriptional machinery as well as with splicing factors (Knoop and Baker, 2000 and references therein), suggesting that they affect the control of gene expression at multiple levels.

Overexpression of unused, non-mutated \textit{ETS} factors by proviral insertional mutagenesis is a recurrent event in erythroleukemia induced in the mouse by the spleen focus forming virus (SFFV) and Friend murine leukemia virus (F-MuLV) components of the Friend virus complex (for a review see Ben-David and Bernstein, 1991). While SFFV-induced erythroleukemia is associated with the recurrent activation of \textit{Spi-1/PU1} expression, F-MuLV-induced erythroleukemia is associated with the activation of \textit{FLI-1} (Ben-David \textit{et al.}, 1991).

To analyze the consequences of the enforced expression of \textit{FLI-1} on erythroid differentiation, we have used a primary erythroblast system. Expression of a temperature-sensitive (ts) version of the v-Sea tyrosine kinase in chicken bone marrow cells results in the selective amplification of pro-erythroblasts (Knight \textit{et al.}, 1988). Upon shift to the non-permissive temperature, ts-v-Sea erythroblast clones are induced to differentiate terminally in response to erythropoietin (Epo) and to die by apoptosis upon Epo deprivation, thereby recapitulating the normal response of erythroblasts to Epo. Using this system, we have shown previously that \textit{FLI-1} profoundly modifies the response of erythroblasts to this cytokine (Pereira \textit{et al.}, 1999), inhibiting their differentiation and simultaneously inducing their proliferation. Similarly, enforced expression of \textit{FLI-1} in a murine erythroblastic cell line alters the normal response of these cells to Epo, promoting their self-renewal rather than their maturation (Tamir \textit{et al.}, 1999).

Besides its inhibitory effect on Epo-induced terminal differentiation, enforced expression of \textit{FLI-1} in primary erythroblasts also inhibits the apoptotic cell death normally induced in these cells upon Epo withdrawal, a property that is associated with up-regulation of \textit{BCL-2} gene expression (Pereira \textit{et al.}, 1999). \textit{BCL-2} is the founding member of a large family of proteins that play a central role in the regulation of apoptosis. The \textit{BCL-2} family includes both anti-apoptotic proteins, such as \textit{BCL-2} itself and \textit{BCL-X\textsubscript{L}}, and pro-apoptotic proteins. The latter include proteins related to \textit{BCL-2}, such as BAX
and a series of proteins which, except for a conserved BH3 domain essential for their pro-apoptotic properties, show little resemblance to BCL-2 (for a review see Strasser et al., 2000). Pro- and anti-apoptotic BCL-2 family members form complexes of unknown stoichiometry in vivo, and the relative ratio of these two classes of proteins has been proposed to determine whether cell survival is favored over cell death.

We show here that induction of BCL-2 expression in FLI-1-transformed erythroblasts occurs at the transcriptional level and that this effect is partially mediated by the binding and activation of BCL-2 promoter sequences by FLI-1. Study of FLI-1 mutants as well as the use of pharmacological inhibitors of BCL-2 further show that up-regulation of BCL-2 expression is important for FLI-1 to promote cell survival of primary erythroblasts and F-MuLV-derived erythroleukemic cell lines. Finally, constitutive expression of BCL-2 itself in primary erythroblasts is shown to be sufficient to promote their survival and to allow their terminal differentiation in the absence of Epo.

Results

FLI-1 up-regulates BCL-2 transcription in erythroblasts

A major contribution of Epo to erythrocytic differentiation is to suppress the apoptotic cell death program of committed erythroid progenitors (Koury and Bondurant, 1988). We previously reported that the enforced expression of FLI-1 in primary erythroblasts both induced the expression of BCL-2 and delayed the apoptotic response of these cells to Epo withdrawal (Pereira et al., 1999). This suggested that up-regulation of BCL-2 expression could be instrumental in the extended survival of FLI-1-transformed erythroblasts under these conditions. FLI-1 being a transcription factor, we analyzed whether BCL-2 up-regulation was the result of the activation of its transcription and whether FLI-1 was directly involved in BCL-2 deregulation.

To analyze whether the up-regulation of BCL-2 mRNA expression observed in FLI-1-transformed erythroblasts occurred at the transcriptional level, run-on assays were performed using nuclei prepared from control and FLI-1-transformed erythroblasts. 32P-labeled run-on transcripts were hybridized to immobilized cDNA fragments of chicken BCL-2, chicken β-actin (as positive control) and human FLI-1 (as specificity control). Hybridization to identical amounts of plasmid DNA was used as a negative control. The results in Figure 1 show that BCL-2 transcription was below detectable levels in control erythroblasts and clearly activated in FLI-1-transformed clones. As expected, transcription of the exogenous human FLI-1 transgene was only detected in FLI-1-transformed erythroblasts, whereas both control and FLI-1-transformed cells transcribed the β-actin gene at a similar level.

The BCL-2 promoter contains FLI-1 response elements and is regulated by FLI-1

The promoters of chicken, mouse and human BCL-2 have only been partially characterized structurally and functionally. Transcriptional initiation of the human BCL-2 gene occurs both from a TATA box-containing promoter (P2) located immediately 5′ to the open reading frame (ORF) encoded by exon 2 and from a TATA-less, upstream P1 promoter (Seto et al., 1988). These features as well as blocks of sequence homology are conserved in mouse and chicken BCL-2 genes (Negroni et al., 1987; Eguchi et al., 1992; Frampton et al., 1996; see Figure 3A). Inspection of the published sequences of these promoters identified several potential EBSs in a conserved region extending between the P1 and P2 transcriptional start sites.

To analyze whether FLI-1 is actually bound to BCL-2 promoter sequences in FLI-1 erythroblasts, we used a chromatin immunoprecipitation assay (ChIP). Erythroblasts expressing a hemagglutinin (HA)-tagged form of human FLI-1 (EpoR/FLI-1) as well as control EpoR erythroblasts were maintained in the absence of hEpo for 16 h at 42°C. Chromatin was prepared from both samples, and the same amount of chromatin (Figure 2A) was subjected to immunoprecipitation using either a monoclonal anti-HA antibody, an anti-FLI-1 antibody or an anti-εzrin antibody as control. The presence of BCL-2 promoter sequences in chromatin immunoprecipitates was analyzed by semi-quantitative PCR using a pair of primers specific for the BCL-2 promoter region extending between P1 and P2. The expected 320 bp BCL-2 promoter fragment was detected specifically in the anti-HA- and anti-FLI-immunoprecipitated chromatin obtained from FLI-1-transformed erythroblasts but not in that obtained from control cells (Figure 2B, lanes 1–4). In contrast, no BCL-2 promoter fragment was detected in the control immunoprecipitation using the anti-εzrin antibody (Figure 2B, compare lanes 5 and 6). In line with the 500–1000 bp size of the chromatin DNA samples, PCR analysis did not detect FLI-1 binding to BCL-2 sequences using a pair of primers specific to the 3′ region of the BCL-2 locus. We conclude from these experiments that BCL-2 promoter sequences are bound by the FLI-1 oncprotein in transformed erythroblasts.

In order to analyze whether FLI-1 can bind the potential EBSs found in the chicken BCL-2 promoter, the corresponding synthetic double-stranded oligonucleotides were analyzed for their ability to bind FLI-1 by competitive
electrophoretic mobility shift assays (EMSAs). As shown in Figure 3B, in vitro translated FLI-1 efficiently bound a 32P-labeled oligonucleotide probe corresponding to a high-affinity EBS (Figure 3B, compare lanes 1 and 2). This retarded complex corresponds to specific binding to DNA since its formation was inhibited by an excess of the same unlabeled oligonucleotide used as competitor but not by the same excess of a mutant oligonucleotide carrying a GG to CC transversion in its GGA core sequence (Figure 3B, lanes 3–6). Unlabeled oligonucleotides encompassing three of the BCL-2 promoter EBSs matches were found specifically to compete the binding of FLI-1 to the radioactive probe, albeit with a lower efficiency than the high-affinity EBS (Figure 3B, lanes 7–12; see Figure 3A for localization of these EBSs in BCL-2 promoter sequences).

To determine whether FLI-1 could transactivate the BCL-2 promoter as the result of its specific binding to DNA, we constructed reporter plasmids in which the luciferase gene was placed under the control of 312 nucleotides of either the wild-type chicken BCL-2 promoter or of a mutant of this promoter in which the FLI-1-binding sites identified by EMSA were mutated in their GGA core. Co-transfection of the wild-type reporter together with a FLI-1 expression vector resulted in a dose-dependent activation of promoter activity (Figure 4B, left panel). In contrast, the mutant reporter construct was not transactivated by FLI-1 (Figure 4B, right panel). Two transcriptional activation domains have been identified in FLI-1 that map N- (ATAD) and C-terminally (CTAD) with respect to the ETS domain (Rao et al., 1993; Baillie et al., 1994; see Figure 4A). Mutant FLI-1 proteins with a deletion in either or both activation domains were generated and analyzed for their ability to transactivate the BCL-2 reporter construct (Figure 4C). As expected, deletion of both activation domains in FLI-1(276–373) generated a transcriptionally defective protein. In contrast, FLI-1 proteins deleted in either the ATAD [FLI-1(225–452)] or the CTAD [FLI-1(1–373)] transactivated the BCL-2 promoter. Taken together, these results show that FLI-1 can transactivate the BCL-2 promoter in a manner dependent upon its tethering to specific promoter sequences and upon the integrity of its transactivation domains.
**Induced expression of BCL-2 in FLI-1-transformed erythroblasts is instrumental in their enhanced survival in the absence of Epo**

To analyze whether up-regulation of BCL-2 is involved in enhanced survival of FLI-1-transformed erythroblasts, we first tested the ability of FLI-1 mutants to induce both erythroblast survival and expression of endogenous BCL-2. Clones of erythroblasts expressing mEpoR or co-expressing mEpoR together with either wild-type FLI-1 (wtFLI-1), FLI-1(225–452) or FLI-1(276–373) were generated. These clones expressed the same amount of mEpoR and the expected FLI-1 protein as assessed by western blot analyses using either an EpoR-specific antiserum or a pan-ETS monoclonal antibody (Figure 5A).

To compare the survival-inducing properties of the respective FLI-1 proteins, erythroblast clones were shifted at 42°C in the absence of hEpo and analyzed for the presence of apoptotic cells by TUNEL assay. In line with previously published results (Tran Quang et al., 1997), mEpoR-expressing control erythroblasts undergo apoptosis as soon as 1 day after Epo deprivation, as shown by their high level accumulation of TdT-positive cells (Figure 5B).

In the same way, mEpoR/FLI-1(276–373) erythroblasts also showed 65–80% TdT-positive cells 24 h after shift, depending on the clone considered, and showed only cell debris when maintained in culture for 3 days under these conditions (Figure 5C). In contrast, both mEpoR/wtFLI-1 and mEpoR/FLI(225–452) erythroblasts showed enhanced survival in these conditions since only 30–50% of the cells were TdT-positive 24 h after shift (Figure 5B). This, together with the fact that wtFLI-1 and FLI-1(225–452) erythroblasts are also induced to proliferate under these conditions (Figure 5D), results in the presence of large amounts of live cells in these cultures after 3 days (Figure 5C). We next compared expression of BCL-2 in control and FLI-1 erythroblasts. Expression of the 6.5 kb BCL-2 mRNA was activated both in wtFLI-1 and FLI-1(225–452) erythroblasts as compared with control cells (Figure 6A). In contrast, no BCL-2 was detected in FLI-1(276–373)-expressing erythroblasts (Figure 6A).

Importantly, activation of BCL-2 expression at the mRNA level was paralleled by the induction of the BCL-2 protein as analyzed by western blot using a BCL-2-specific antibody (Figure 6B). These studies show that the ability of FLI-1 to prolong cell survival in the absence of hEpo is associated with its ability to up-regulate endogenous BCL-2 expression in erythroblasts, a fact that correlates with its ability to transactivate the BCL-2 promoter in transient transfection experiments.

To assess further the importance of BCL-2 expression in FLI-1-mediated inhibition of apoptosis, we used a recently described pharmacological inhibitor of BCL-2/BCL-XL (Tzung et al., 2001). FLI-1 erythroblasts were shifted to 42°C in the absence of hEpo and treated with either the active 2-methoxy-antimycin A3 or the inactive antimycin A3 phenacyl ether derivative. As shown in Figure 7, FLI-1-transformed erythroblasts died when treated with 2-methoxy-antimycin A3, whereas they were not affected by the addition of either dimethylsulfoxide (DMSO) or the same amount of antimycin A3 phenacyl ether. This indicates that integrity of the BCL-2 pathway is required for the survival of FLI-1-transformed erythroblasts.

**Fig. 4.** FLI-1 transactivates the BCL-2 promoter. (A) Schematic representation of the FLI-1 deletion mutants used. ATAD, N-terminal transactivation domain; CTAD, C-terminal transactivation domain. (B) Transactivation of the BCL-2 promoter requires tethering of FLI-1 to promoter sequences. QT6 cells were co-transfected with increasing amounts of the ΔEβ HA-FLI-1 expression vector (0.2, 0.4, 0.8 and 1.6 μg) along with either 0.2 μg of the –312 BCL-2-Luc reporter plasmid (left panel) or 0.2 μg of the –312 BCL-2-Luc reporter in which the three EBSs have been mutated in their core sequence (right panel). Luciferase activity (relative light units) was evaluated in cell extracts and normalized relative to the β-galactosidase activity encoded by a co-transfected LacZ expression vector. Bottom tracks: FLI-1 expression in transfected cells as detected by western blotting analyses.

(C) Transactivation of the BCL-2 promoter is dependent on the integrity of FLI-1 transcriptional activation domains. A 0.2 μg aliquot of the –312 BCL-2-Luc reporter plasmid was co-transfected along with increasing amounts (0.4, 0.8 and 1.6 μg) of the ΔEβ expression plasmid encoding wtFLI-1, FLI-1(225–452), FLI-1(1–373) or FLI-1(276–373). Luciferase activity was evaluated as described above. Bottom tracks: expression of FLI-1 mutants detected by western blotting using the anti-pan ETS antibody.
Finally, we asked whether constitutive expression of BCL-2 would be sufficient to inhibit apoptosis of primary erythroblasts maintained in the absence of hEpo. A series of erythroblast clones expressing either mEpoR or co-expressing mEpoR and human BCL-2 were generated. These clones expressed similar levels of EpoR, whereas mEpoR/BCL-2 clones expressed, in addition, the expected 24 kDa BCL-2 protein (Figure 8A and data not shown). As described above, mEpoR clones maintained in the absence of hEpo rapidly died by apoptosis (Figure 8B), with no live cells detectable in these cultures after 3 days (Figure 8C, top panels). In contrast, mEpoR/BCL-2 erythroblasts showed a clearly extended survival under these conditions (Figure 8B and C). Moreover, they differentiated terminally, as evidenced over time by reduction of their cell size, by their acquisition of the oval-shaped morphology of mature erythrocytes and by their accumulation of hemoglobin as assessed by benzidine staining (Figure 8C). We conclude that the enforced expression of BCL-2 is sufficient to bypass the requirement for Epo to promote survival and terminal differentiation of primary erythroblasts.
**BCL-2 is a FLI-1 target in erythroblasts**

**Fig. 6.** Analysis of BCL-2 expression in control and erythroblast clones expressing wild-type or mutant FLI-1. (A) Northern blot analysis. Poly(A)+ RNA from EpoR control erythroblasts or EpoR erythroblasts expressing either wtFLI-1 or the indicated FLI-1 mutants was isolated 24 h after the cells had been shifted to 42°C in the absence of hEpo. Hybridization was carried out with a probe to chicken BCL-2 (top panel) or chicken Band 4.1 as loading control (lower panel). The 6.6 kb Band 4.1 transcript and the 6.5 kb BCL-2 transcript are indicated by arrowheads. (B) Western blot analysis. The indicated erythroblast clones were maintained for either 24 or 48 h at 42°C in the absence of hEpo. Cells were counted and the same number of live cells lysed. Cell lysates were analyzed on a 15% acrylamide gel in the presence of SDS, followed by transfer and immunoblotting with an antibody to chicken BCL-2 (upper panels) or an antibody to viral p27 as loading control (lower panels). The 26 kDa BCL-2 protein and 27 kDa p27 protein are indicated by arrowheads.

**Fig. 7.** BCL-2 function is required for survival of FLI-1-transformed erythroblasts. The indicated wtFLI-1-expressing erythroblast clones were maintained at 42°C in the absence of hEpo and treated with 10 μg/ml of either 2-methoxy antimycin A3 (Me-Antimycin) or antimycin A3 phenacyl ether (AntimycinA Phe), or with DMSO. At 6 h after treatment, cells were counted using Trypan Blue to quantify cell death.

**Fig. 8.** Constitutive BCL-2 expression induces cell survival and terminal differentiation of primary chicken erythroblasts. Ts-x-Sea erythroblast clones expressing either EpoR or both EpoR and hBCL-2 were generated by retroviral-mediated gene transfer. (A) Western blot analysis of exogenous hBCL-2 and EpoR expression using anti-hBCL-2 antibody and anti-EpoR antibody. One representative clone of each combination is shown. (B) EpoR and EpoR/hBCL2 erythroblast clones were maintained at 42°C in the absence of hEpo for 24 h and processed for quantitative evaluation of cell survival by TUNEL assay. (C) EpoR and EpoR/hBCL2 erythroblast clones were maintained at 42°C in the absence of hEpo for 1, 2, 3 or 4 days. Aliquots of cells were cytocentrifuged onto slides, and stained with neutral benzidine (stains hemoglobin in brown) and Giemsa.

Consistent with recently published results (Howard et al., 2001), cell lines derived from F-MuLV-induced erythroleukemia, which express high levels of FLI-1, also displayed high levels of BCL-2 RNA and protein (Figure 9A and B). In contrast, cell lines derived from SFFV-induced erythroleukemia, which contain significantly lower levels of FLI-1, express low amounts of BCL-2, only detectable by RT–PCR (Figure 9A). This raises the possibility that induction of BCL-2 expression is dependent upon a threshold level of FLI-1 expression. To analyze this point in further detail, we expressed HA-tagged FLI-1 using a murine stem cell virus (MSCV)-FLI-1–enhanced green fluorescent protein (eGFP) transgene in IW-9, a cell line derived from a F-MuLV-induced erythroleukemia which contains low levels of FLI-1 protein (data not shown) and barely detectable levels of BCL-2 (Figure 9C and D). IW-9 cells transduced with an MSCV–eGFP transgene were used as control. Single eGFP-positive clones were obtained by limiting dilution, and FLI-1-expressing and control clones were compared

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for **BCL-2** expression. As shown in Figure 9C and D, enforced expression of **HA-FLI-1** in **IW-9** cells resulted in the up-regulation of **BCL-2** expression at both the RNA (Figure 9C) and protein levels (Figure 9D). These results show that up-regulation of **BCL-2** expression in F-MuLV-derived mouse erythroleukemic cells depends upon a threshold level of **FLI-1** protein.

**Discussion**

The activation of the oncogenic properties of **BCL-2** in follicular lymphoma carrying a t(14;18) chromosomal translocation together with the demonstration that **BCL-2** overexpression in mouse B cells both induces lymphoma and protects these cells from apoptosis has led to the notion that an increase in apoptotic threshold is a major component in tumorigenesis (for a review see Adams and Cory, 1998). Since then, it has become clear that a recurrent theme in leukemia and solid tumor progression is the mutation or deregulated expression of components of the **BCL-2** pathway. However, in only a few instances have the molecular mechanisms underlying the deregulated expression of **BCL-2** family members been characterized. For example, oncogenic tyrosine kinases such as **BCR-ABL** result in the induction of both **BCL-2** (Sanchez-Garcia and Grutz, 1995) and **BCL-X** expression, the latter through STAT-mediated activation of its promoter (Gesbert and Griffith, 2000). Transcription factors of the Myb family play a role in **BCL-2** up-regulation in several normal and leukemogenic processes (Frampton et al., 1996; Taylor et al., 1996). Sporadic Wilm’s tumors express high levels of **BCL-2**, a property that results at least in part from transcriptional activation of **BCL-2** gene transcription by WT1 (Mayo et al., 1999).

Our results show that up-regulation of **BCL-2** expression following the enforced expression of the **FLI-1** oncprotein in primary avian erythroblasts occurs at the transcriptional level and is mediated, at least in part, by the direct activation of the **BCL-2** gene promoter by **FLI-1**. A similar scenario is likely to occur in the course of F-MuLV-induced erythroleukemia. First, the vast majority of primary F-MuLV-induced leukemia and derived cell lines express high levels of both **FLI-1** and **BCL-2** protein and RNA (Howard et al., 2001; this study). Secondly, in these cell lines (our unpublished data), as in **FLI-1**-transformed primary avian erythroblasts, **FLI-1** is found by ChIP analyses to be bound to **BCL-2** promoter sequences. Thirdly, **FLI-1** can transactivate a mouse **BCL-2** promoter reporter construct in transient transfection assays (our unpublished data). Finally, **BCL-2** gene expression can be induced following enforced expression of exogenous **FLI-1** in a rare F-MuLV-derived cell line expressing low levels of **FLI-1**. Progression of F-MuLV-induced erythroleukemia is associated with up-regulation of **BCL-2** expression (Howard et al., 2001), suggesting that **BCL-2** gene regulation is controlled at several levels in **FLI-1** transformed erythroblasts. ETS proteins usually regulate transcription in cooperation with other cis-bound factors through the coordinate assembly of large multi-protein complexes (Hernandez-Munain et al., 1998; and references therein). The **BCL-2** promoter can be regulated by several transcription factors that can functionally interact with ETS factors, including members of the CREB/ATF family, **Sp1** and **p53** (Gegonne et al., 1993; Hernandez-Munain et al., 1998; Sampath et al., 2001). **BCL-2** transcription in F-MuLV-induced erythroleukemia is therefore likely to depend upon the interaction between overexpressed **FLI-1** and cofactors, the expression or activity of which may vary during disease progression. The fact that induction of **BCL-2** expression appears to require a threshold level of **FLI-1** may reflect the requirement for a large amount of **FLI-1** protein either to bind the **BCL-2** promoter ETSs efficiently and/or to establish productive contacts with cis-bound cofactors.

The role of the **BCL-2** protein family in red blood cell development is only partly understood. Several **BCL-2** family members are expressed in primary erythroblasts, including **BCL-XL**, **MCL1**, **BAX** and **BAD**, but not **BCL-2** itself (Gregori and Bondurant, 1997). In line with an undetectable level of its expression in primary erythroblasts, **BCL-2**-deficient mice show no defect in
erythropoiesis (Veis et al., 1993). In contrast, BCL-XL is strongly up-regulated during Epo-induced differentiation (Gregoli and Bondurant, 1997; Gregory et al., 1999), and genetic studies of BCL-XL–/– mice have demonstrated a specific, non-redundant role for BCL-XL at the end of the differentiation process (Motoyama et al., 1999; Wagner et al., 2000). Whether BCL-XL or other anti-apoptotic BCL-2 family members are part of the survival pathway(s) elicited early during Epo-induced differentiation is unknown. It is therefore unclear at present whether the activation of BCL-2 expression in FLI-1-transformed erythroblasts augments an anti-apoptotic pathway normally activated by Epo early in erythroid differentiation or whether it corresponds to the implementation of a de novo pathway in these transformed cells.

Several lines of evidence indicate that up-regulation of BCL-2 expression is involved in the survival-inducing properties of FLI-1-transformed erythroblasts. First, a correlation was found between the ability of wtFLI-1 and FLI-1 mutants to induce both cell survival and endogenous BCL-2 expression in vivo. Secondly, inhibitor studies show that under experimental conditions in which survival of transformed erythroblasts is critically dependent upon FLI-1, BCL-2 function is required for optimal survival of these cells. Importantly, this inhibitor also induces apoptosis of F-MuLV-derived erythroleukemic cell lines (our unpublished observations), indicating that the survival of these cells also depends on the activity of a BCL-2-dependent pathway. Thirdly, ectopic expression of BCL-2 in primary erythroblasts bypasses their requirement for Epo to allow their survival in tissue culture. These data strongly suggest that deregulated expression of BCL-2 is an important determinant at one or several stages of F-MuLV-induced erythroleukemia. The fact that enforced expression of BCL-2 in contrast to FLI-1 does not inhibit erythroid differentiation implies that FLI-1 deregulates pathways distinct from BCL-2 up-regulation to block differentiation.

EpoR signaling is essential to red blood cell development since disruption of either EpoR or Epo genes in mice results in severe deficiency of definitive type erythropoiesis (Wu et al., 1995; Lin et al., 1996). Fetal liver cells of these mice contain normal numbers of BFU-E and CFU-E, but these cells failed to mature further to generate terminally differentiated erythrocytes. Our results show that the provision of a strong survival signal by enforced expression of BCL-2 in primary erythroblasts bypassed the requirement of these cells for Epo to induce their terminal differentiation. This suggests that the main function of Epo/EpoR in erythroblast differentiation is not instructive, but rather critically involved in the activation of signaling pathway(s) essential to cell survival. This conclusion is in line with the observation that activation of cytokine receptors unrelated to the EpoR can fully replace Epo and rescue the erythroid maturation defect of EpoR–/– fetal liver cells (Socolovsky et al., 1998). They apparently contrast, however, with the observation that transgenic or retrovirally induced expression of BCL-2 failed to replace Epo in its ability to induce colonies from BFU-E and CFU-E (Lacronique et al., 1997; Chida et al., 1999). However, besides its anti-apoptotic function, Epo also has mitogenic properties (Spivak et al., 1991) that are essential for CFU-E and BFU-E colony formation. Our results in liquid cultures indicate that Epo-mediated survival signals are largely sufficient to allow erythroblasts to respond to a genetically pre-determined differentiation program, whereas a distinct pathway(s) is involved in Epo-induced proliferation.

Materials and methods

DNA constructs and generation of recombinant retroviruses

The pC1a12, ΔEB-HA, pRCAS-A, pRCAS-mEpoR, pCRNCM-mEpoR, pSFCV and pSFCV-FLI-1(1–452) encoding an HA-tagged version of wild-type hFLI-1 have been described previously (Tran Quang et al., 1997; Pereira et al., 1999), pRCAS-hBCL-2 was kindly provided by D.Ewert (Wistar Institute). To generate pSFCV-FLI-1(225–452), ΔEB-FLI-1(225–452) (Baillie et al., 1994) was digested with BglII and the insert subcloned into a BglII-restricted pC1a12 adaptor plasmid and next subcloned into ClaI-restricted pSFCV. To generate pSFCV-FLI-1(276–373), the corresponding region of the FLI-1 cDNA was PCR amplified, using 5’-GGCTTCAGGCGCTGGAAAGGGATACCT-3’ and 5’-CCACGCTTCTACAGTGACTGGTGAGACCC-3’ primers. The XhoI-HindIII-digested PCR fragment was inserted into similarly restricet ΔEB-HA, resulting in the in-frame fusion of FLI-1 sequences with those encoding the HA epitope. The fragment encoding HA-FLI-1(276–373) was subcloned into EcoRI–HindIII-digested pC1a12 and next into ClaI-restricted pSFCV. To generate MSCV-FLI-1, pSFCV-FLI-1(1–452) was digested with EcoRV and the insert subcloned into HpaI-digested MSCV–eGFP (a gift of Dr H.Singh, University of Chicago). The reporter plasmid driving luciferase expression from the –12 to +3 chicken BCL-2 promoter has been described previously (Frampton et al., 1996). The BCL-2 promoter carrying mutations in the three EBSs was created by PCR, by amplification of a 333 bp HindIII–XhoI fragment using 5’-GGAAGAAGTTGCACGGAGGCAGG-3’ (mutation at EBS site at –301) and 5’-CCCTCTAGACATGTCTCCGACCAAGCGGAGATGAGG-3’ (mutation of EBSs at –37 and –12) as mutagenic primers. All inserts were sequenced completely to verify their identity.

Infectious avian retroviruses were generated as previously described (Tran Quang et al., 1997). Helper-free MSCVs were produced following co-transfection of the Phoenix/Eco cell line (a gift of Dr G.Nolan, Stanford University) with the respective MSCV derivatives using the calcium phosphate co-precipitation method.

Cell culture, retroviral infection, differentiation and survival assays

The QT6 quail fibroblast cell line and CEF were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% chicken serum and 1 mM glutamine. Friend erythroleukemia cell lines CB3, CB7, HB22.2, DP16-1, DP17, DP18 (a gift from Dr Y.Ben-David, Toronto) and the I9W cell line (a gift from Dr F.Wendling, Paris) were cultivated in Iscove’s modified Eagle’s medium (IMDM) supplemented with 10% FBS. To transduce I9W cells with helper-free MSCV, cells were plated on a retinobcin-coated Petri dish and incubated with the respective viral supernatants. After 48 h, cells were plated in 96-well plates under limiting dilution conditions and GFP-positive clones amplified.

Primary erythroblast clones expressing the respective exogenous proteins were obtained as previously described (Tran Quang et al., 1997). Erythroblasts were expanded in CFE medium containing 10% fetal calf serum (FCS) and 1.4 mM insulin in the presence of I5-F (Novo Nordisk) and analyzed by western blot for the expression of the expected exogenous proteins. Differentiation analyses of erythroblasts and TUNEL assays were performed as described previously (Tran Quang et al., 1997).

RNA expression analysis

Total RNA was isolated using the RNeasy Midi Kit (Qiagen). Poly(A)+ mRNAs were purified using the PolyAT tract mRNA Isolation System (Promega). Poly(A)+ RNA samples (10 µg) were analyzed for the expression of specific genes either by northern blot as previously described (Pereira et al., 1999) or by RT–PCR. cDNAs were synthesized using the First Strand cDNA Synthesis kit (Amersham) using the manufacturer’s Noril-d(T)20 primers. The generated cDNA products were amplified using Taq polymerase and the following primers: mBCL-2: 5’-TGTCACAGGCGGCTACGG-3’ and 5’-GGGCGGTATGGTGC-CACCAG-3’. mβ-Actin: 5’-GTTGGCCGCCCTAGGCCACACG-3’ and
Nuclear run-on assays

Erythroblasts were resuspended at a concentration of 2 × 10⁸ cells/ml in 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 2 mM MgCl₂, and lysed by adding an equal volume of 50 mM Tris–HCl pH 7.0 containing 1% (v/v) NP-40 and by incubating the mix for 7 min at 4°C. Cell lysis was followed by phenol/chloroform microscopy and nuclei were resuspended at a concentration of 2 × 10⁶ nuclei/ml in a solution of 50 mM Tris–HCl pH 8.3, 5 mM MgCl₂, 0.1 M EDTA pH 7.0, 40% (v/v) glycerol and stored at –80°C. A total of 2.4 × 10⁶ nuclei were incubated at 30°C for 30 min in 200 µl of 5 mM Tris–HCl pH 7.5, 2.5 mM MgCl₂, 150 mM KCl, 0.25 mM ATP, 0.25 mM CTP, 0.25 mM GTP, 280 µM of [α-3²P]UTP (>3000 Ci/mmol; Amersham) and 120 U of RNasin (Boehringer Mannheim). The same amount of radioactive counts from each sample of 3²P-labeled run-on RNA were hybridized as described previously (Smith et al., 1998) to 1 µg of target sequences dot-blotted onto a nylon membrane (Positive TM Membrane, Qiagen). Target sequences were: avian BCL-2 cDNA fragment (nucleotides 1–702 encoding the ORF); avian β-actin cDNA fragment (nucleotides encoding amino acids 1–288); human FL1 cDNA fragment (nucleotides encoding amino acids 1–234); a 625 bp KpnI–NaeI fragment of pBluescript KS−; and a 692 bp Drel fragment of pUC19. After hybridization, membranes were washed three times for 10 min in 2× SSC, 0.1% SDS at 25°C and twice for 15 min in 0.2× SSC, 0.1% SDS at 60°C. Radioactive images were analyzed using a PhosphorImager.

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