Direct regulation of *BCL-2* by *FLI-1* is involved in the survival of *FLI-1*-transformed erythroblasts

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Rearrangement of the *FLI-1* locus with ensuing over-expression of *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 *BCL-2*. We further show that *FLI-1* binds *BCL-2* promoter sequences in transformed erythroblasts, and *in vitro* studies identify specific *FLI-1*-binding sites essential for the transactivation of the *BCL-2* promoter by *FLI-1*. Analysis of *FLI-1* mutants showed a correlation between the ability of *FLI-1* to transactivate *BCL-2* promoter sequences and their ability to inhibit apoptosis in the absence of Epo. Moreover, inhibitor studies confirmed the essential role of *BCL-2* for *FLI-1*-transformed erythroblast survival. Finally, enforced expression of *BCL-2* was sufficient to promote survival and terminal differentiation of erythroblasts in the absence of Epo. These results show that *BCL-2* is an *in vivo* target of *FLI-1* in *FLI-1*-transformed erythroblasts and that its deregulated expression is instrumental in the survival of these cells.

Keywords: *BCL-2*/erythroblast survival/*FLI-1*/Friend erythroleukemia

Introduction

The *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 Boureux, 1997). The *ETS* domain of these proteins binds DNA response elements (EBS) in the regulatory regions of *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 *ETS* proteins with unrelated transcriptional regulators.

ETS proteins play a central role in a variety of oncogenic processes. Transformation by signal transduction oncogenes such as *RAS* is critically dependent upon

ETS proteins (Wasylyk *et al.*, 1994). Moreover, several solid tumors and leukemias are linked specifically to mutation or abnormal expression of *ETS* proteins. For example, the hallmark of Ewing sarcoma is the fusion of the 5' half of the *EWS* gene to the 3' part of one of several *ETS* genes, most often *FLI-1* or *ERG*, as the result of specific chromosomal translocations (Delattre *et al.*, 1992). The chimeric proteins encoded by the *EWS-FLI-1*/*EWS-ERG* fusion oncogenes are aberrant transcriptional regulators of EBS-driven transcription (Bailly *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 unfused, non-mutated *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 *Spi-1*/*PU1* expression, F-MuLV-induced erythroleukemia is associated with the activation of *FLI-1* (Ben-David *et al.*, 1991).

To analyze the consequences of the enforced expression of *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 *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 *FLI-1* profoundly modifies the response of erythroblasts to this cytokine (Pereira *et al.*, 1999), inhibiting their differentiation and simultaneously inducing their proliferation. Similarly, enforced expression of *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 *et al.*, 1999).

Besides its inhibitory effect on Epo-induced terminal differentiation, enforced expression of *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 *BCL-2* gene expression (Pereira *et al.*, 1999). *BCL-2* is the founding member of a large family of proteins that play a central role in the regulation of apoptosis. The *BCL-2* family includes both anti-apoptotic proteins, such as *BCL-2* itself and *BCL-X_L*, and pro-apoptotic proteins. The latter include proteins related to *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

Fig. 1. *BCL-2* transcription is induced in FLI-1-transformed erythroblasts. Run-on experiments were carried out on nuclei isolated from either control or HA-FLI-1-transformed ts-v-Sea erythroblast clones shifted for 1 h to 42°C in the absence of hEpo. Bottom panel: the run-on reaction was performed in the presence of 4 μg/ml α-amanitin. Equivalent amounts of radioactive counts of ³²P-labeled run-on RNA were hybridized to chicken *BCL-2* cDNA. A fragment of chicken β -actin and fragments derived from pUC and pBluescript plasmids were used as positive and negative controls, respectively. A 752 bp fragment of human FLI-1 cDNA was used as an internal specificity control.

(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 (Negrini *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-ezrin 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-1-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-ezrin 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 (data not shown). We conclude from these experiments that *BCL-2* promoter sequences are bound by the FLI-1 oncoprotein 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

Fig. 2. FLI-1 binds *BCL-2* promoter sequences *in vivo*. Control and FLI-1-transformed ts-v-Sea erythroblast clones were maintained for 16 h at 42°C in the absence of hEpo. Cells were fixed and their chromatin isolated and processed as indicated. (A) Direct PCR amplification of chromatin obtained from control and FLI-1-expressing erythroblast clones, using primers specific to the P2 promoter region of *BCL-2* (input analysis). The expected 320 bp *BCL-2* promoter fragment is indicated by a black arrow. (B) PCR amplification of immunoprecipitated chromatin fragments obtained from control and FLI-1-expressing erythroblast clones using anti-HA, anti-FLI-1 or control anti-ezrin antibodies. PCR on 100 ng of genomic DNA isolated from chicken ts-v-Sea erythroblasts is included as a positive control. The 320 bp *BCL-2* promoter fragment is indicated by a black arrow.

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

Fig. 3. Identification of FLI-1-binding sites in the *BCL-2* promoter. (A) Schematic representation of the chicken *BCL-2* promoter. The P2 and P1 transcriptional initiation sites are indicated. The intron between the first, non-coding exon and the second exon is shown as a hatched box. The initiator methionine encoded by exon 2 is indicated. Sequence of the 312 bp *BCL-2* promoter located upstream of the initiating ATG is shown. The FLI-1-binding sites identified by EMSA are underlined. (B) Competitive EMSA was performed with *in vitro* translated FLI-1 and 100 fmol of a consensus FLI-1-binding site oligonucleotide probe. In lanes 3–12, a 150- and 300-molar excess of unlabeled oligonucleotide competitor was added as indicated. EBS, oligonucleotide competitor corresponding to the probe; EBS mut, mutant oligonucleotide competitor carrying a GG to CC transversion in its GGA core sequence. Oligonucleotides encompassing three of the *BCL-2* promoter EBSs are named 1, 2 and 3 (see A). (C) EMSA was performed as in (B) except that an antibody to FLI-1 (lane 14) or a control antibody (lane 15) were added at the end of the binding reaction.

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; Bailly 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-X_L (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 Δ EB HA-FLI-1 expression vector (0.2, 0.4, 0.8 and 1.6 μ g) along with either 0.2 μ g of the -312 *BCL-2*-Luc reporter plasmid (left panel) or 0.2 μ g 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 μ g aliquot of the -312 *BCL-2*-Luc reporter plasmid was co-transfected along with increasing amounts (0.4, 0.8 and 1.6 μ g) of the Δ EB 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.

Fig. 5. Survival-inducing properties of wtFLI-1 and FLI-1 deletion mutants. (A) Expression of exogenous proteins in the indicated erythroblast clones was analyzed by western blotting using either an anti-EpoR or the anti-pan ETS antibody. Control cells were ts-v-Sea erythroblasts transduced by the empty SFV vector. One representative clone is shown. (B) Quantitative evaluation of apoptosis by TUNEL assay in the indicated erythroblast clones maintained at 42°C for 24 h in the absence of hEpo. Data obtained for three independent clones are shown. (C) Cytochemical analysis of the indicated erythroblast clones maintained at 42°C for 3 days in the absence of hEpo. Cells were stained with neutral benzidine and Giemsa. (D) Proliferation of the indicated erythroblast clones maintained at 42°C in the absence of hEpo. Cells were counted daily using an electronic cell counter (Schärfe System) and maintained at densities between 1.5 and 4×10^6 cells/ml by appropriate dilution every day. Cumulative cell numbers are shown over time.

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.

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.

***BCL-2* is up-regulated specifically in murine erythroleukemic cell lines expressing large amounts of FLI-1**

A number of cell lines have been derived from either F-MuLV- or SFFV-induced erythroleukemia. We therefore analyzed the expression of *BCL-2* in these cells.

Fig. 8. Constitutive *BCL-2* expression induces cell survival and terminal differentiation of primary chicken erythroblasts. Ts-v-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

Fig. 9. *BCL-2* is expressed in murine erythroleukemic cell lines expressing a high level of *FLI-1*. (A) RT-PCR analysis of *BCL-2* mRNA expression in the indicated F-MuLV- and SFFV-derived cell lines. The reverse transcription reaction utilized an oligo(dT) primer. PCR (30 cycles) was carried out using primers specific for either the murine *BCL-2* gene (upper panel) or the β -actin gene as normalization control (lower panel). PCR products were run on a 2% agarose gel containing ethidium bromide. The expected 427 bp *BCL-2* fragment and 447 bp β -actin gene fragment are indicated by black arrows. (B) Western blot analysis of *FLI-1* and *BCL-2* expression in mouse erythroleukemic cell lines. The same number of cells were lysed and proteins separated by electrophoresis on a 15% acrylamide gel containing SDS, transferred onto membrane and immunoblotted successively with an anti-mouse *BCL-2* antibody, an anti-*FLI-1* antibody and an anti-ERK antibody as loading control. The 26 kDa *BCL-2* protein, 50 kDa *FLI-1* protein and 42–44 kDa ERK1/2 proteins are indicated by black arrows. (C) RT-PCR analysis of *BCL-2* expression in a representative IW9 clone transduced with the MSCV-eGFP vector (D5) and a representative IW9 clone transduced with the MSCV-eGFP/HA-*FLI-1* vector (F7). RT-PCR was conducted as described in (A). (D) Western blot analysis of the IW9 clones transduced with either the control MSCV-eGFP vector (clone D5) or with MSCV-eGFP/HA-*FLI-1* (clones B4 and F7). Antibodies used were: an anti-mouse *BCL-2* antibody, a polyclonal anti-HA antibody to detect the exogenously expressed HA-tagged-*FLI-1* protein, and an anti-ERK antibody as loading control. The respective proteins are indicated by black arrows.

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_L* expression, the latter through STAT-mediated activation of its promoter (Gesbert and Griffin, 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 Wilms' 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* oncoprotein 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 multiprotein 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 EBSs 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-X_L*, *MCL1*, *BAX* and *BAD*, but not *BCL-2* itself (Gregoli 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-X_L is strongly up-regulated during Epo-induced differentiation (Gregoli and Bondurant, 1997; Gregory *et al.*, 1999), and genetic studies of BCL-X_L^{-/-} mice have demonstrated a specific, non-redundant role for BCL-X_L at the end of the differentiation process (Motoyama *et al.*, 1999; Wagner *et al.*, 2000). Whether BCL-X_L 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 erythroblast 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 EpoR 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 pCla12, 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) (Bailly *et al.*, 1994) was digested with *Bgl*II and the insert subcloned into a *Bgl*II-restricted pCla12 adaptor plasmid and next subcloned into *Cla*I-restricted pSFCV. To generate pSFCV-FLI-1(276–373), the corresponding region of the FLI-1 cDNA was PCR amplified, using 5'-GCCTCGAGGCTGGAAGCGGCAGATCC-3' and 5'-CCAAGCTTCTACGGATGTGGCTGCAGAGCC-3' as primers. The *Xho*I-*Hind*III-digested PCR fragment was inserted into similarly restricted 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 *Eco*RI-*Hind*III-digested pCla12 and next into *Cla*I-restricted pSFCV. To generate MSCV-FLI-1, pSFCV-FLI-1(1–452) was digested with *Eco*RV and the insert subcloned into *Hpa*I-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 *Hind*III-*Xho*I fragment using 5'-GGGAAGCTTGACAGCCAGGAG-GAAGCGTTGCTCAAGTAG-3' (mutation at EBS site at -301) and 5'-CCCCTCGAGCATGGTTTCCGAGGCCAAGCAGCGAGTGGGAGG-GGGAGACCAAGG-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 IW9 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 IW9 cells with helper-free MSCV, cells were plated on a retromycin-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 CFU-E medium containing 100 ng/ml stem cell factor (SCF) and 1.4 nM insulin (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 *Not*I-d(T)₁₈ primers. The generated cDNA products were amplified using *Taq* polymerase and the following primers: *mBCL-2*: 5'-TGTCACAGAGGGGCTACGAG-3' and 5'-GGGCGATGTGTCC-ACCAGG-3'; *mβ-Actin*: 5'-GTGGGCCGCCCTAGGCACCAG-3' and

5'-CTCTTTGATGTACGACGATTTC-3'. After 10 min at 94°C, 30 amplification cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) were performed, followed by a 10 min elongation step at 72°C. PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

Immunoblot analysis

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed (15 µl per 10⁶ cells) with Laemmli sample buffer. For BCL-2 expression, cells (10 µl per 10⁶ cells) were lysed in immunoprecipitation lysis buffer (Tran Quang *et al.*, 1997) and centrifuged at 1000 g for 10 min. The resulting supernatant was diluted with 1 vol. of 2× Laemmli sample buffer. Protein extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Proteins were revealed by ECL (Amersham). Anti-BCL-2 monoclonal antibody (sc-509), anti-mouse EpoR polyclonal antibody (sc-697) and anti-FLI-1 polyclonal antibody (sc-356) were from Santa Cruz Biotechnology. Anti-chicken BCL-2 monoclonal antibody was a kind gift of Dr Y.Eguchi (Osaka University). The anti-pan ETS monoclonal antibody was kindly provided by Dr N.Bhat (Frederick Cancer Research Center). Horseradish peroxidase-conjugated secondary antibodies were from Amersham.

Transient transfections and luciferase assays

Transient transfections were carried out using the calcium phosphate co-precipitation method as previously described (Bailly *et al.*, 1994). Cell lysates were prepared 48 h after transfection and assayed for luciferase activity using the Promega Luciferase Assay System kit. Results were normalized with respect to the β-galactosidase activity expressed from 100 ng of a co-transfected pEF-Bos-LacZ plasmid, using the Galacto-Star kit (Tropix). The results shown represent the average luciferase activity and standard deviation from three independent experiments.

Electrophoretic mobility shift assay

EMSA and supershift analysis were performed as described previously (Bailly *et al.*, 1994), using *in vitro* transcribed-translated FLI-1 protein produced in reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega). The double-stranded wild-type EBS oligonucleotide used corresponds to a high-affinity FLI-1-binding site, (+) strand 5'-TCGGGTTCGACATAACCGGATGTGGGC-3'. The mutant (EBS mut) was 5'-TCGGGTTCGACATAACCCCATGTGGGC-3'. Oligonucleotides designed from the chicken BCL-2 promoter were as follows: 5'-TCGGCCAGGAGGAAGCGTTGC-3' (-301 EBS site); 5'-TCGGGGGAGAGGAAGGAGAGG-3' (-37 EBS site) and 5'-TCGGCCGAGGGGAAGCAGCGA-3' (-12 EBS site).

Chromatin immunoprecipitation assay

Erythroblasts were fixed in 1% formaldehyde for 15 min at 42°C, washed twice in ice-cold PBS and lysed in 50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS (4 × 10⁷ cells per ml). Cross-linked chromatin was sonicated to obtain DNA fragments of 500–2000 bp. After 10 min centrifugation at 14 000 r.p.m. and 4°C, supernatants were diluted 10-fold in 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100 and protease inhibitors as above. This chromatin preparation (1 ml) was incubated for 30 min at 4°C with 80 µl of a 50% protein A-Sepharose slurry pre-equilibrated in 20 mM Tris-HCl pH 8.1, 2 mM EDTA, 20 µg of sonicated salmon sperm DNA and 1 mg/ml bovine serum albumin. After centrifugation, the supernatant was incubated for 90 min at 4°C with the indicated antibody, followed by the addition of 60 µl of pre-equilibrated protein A-Sepharose. After 30 min of incubation with gentle mixing, beads were collected by centrifugation and washed by gentle mixing in 1 ml of the following buffers: (1) 20 mM Tris-HCl pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100; (2) the same as in 1 but with 500 mM NaCl; (3) 10 mM Tris-HCl pH 8.1, 0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1% NaDoc; and (4) twice with 20 mM Tris-HCl pH 8.1, 2 mM EDTA. Complexes were dissociated by two rounds of incubation in 250 µl of 0.1 M NaHCO₃/1% SDS (15 min each). A 20 µl aliquot of 5 M NaCl was added to the pooled eluates and incubation continued for 4 h at 65°C, followed by the addition of 20 µl of Tris-HCl pH 6.5, 10 µl of 0.5 M EDTA and 2 µl of 10 mg/ml proteinase K. After 1 h at 45°C, DNA was recovered by phenol/chloroform extraction and ethanol precipitation using 20 µg of glycogen as carrier. The presence of BCL-2 promoter fragments in immunoprecipitated chromatin was detected by PCR using 5'-CATGTGTTCCGACAGC-CAGG-3' and 5'-CATGGTTTCCGAGGGGAAGC-3' as primers. PCR products were run on 2% agarose gels containing ethidium bromide.

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 phase contrast microscopy and nuclei were resuspended at a concentration of 2 × 10⁸ nuclei/ml in a solution of 50 mM Tris-HCl pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA pH 7.0, 40% (v/v) glycerol and stored at -80°C. A total of 2.4 × 10⁷ 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 µCi 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-spotted onto a nylon membrane (Positive TM Membrane, Q.Biogene). 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 FLI-1 cDNA fragment (nucleotides encoding amino acids 1–234); a 625 bp KpnI-NaeI fragment of pBluescript KS⁺; and a 692 bp DraI 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.

Acknowledgements

The authors thank N.K.Bath, Y.Ben-David, H.Beug, Y.Eguchi, D.Ewert, D.Hockenbery, G.Nolan, H.Singh, F.Wendling and K.Weston for reagents; D.Rouillard and M.Hours for help with FACS analyses; J.Raingaud for help with site-directed mutagenesis; M.Pironin and M.Williams for expert technical assistance; Y.Ben-David and H.Beug for helpful discussions; and Janssen-Cilag for generous supply of recombinant human erythropoietin. I.L. is supported by pre-doctoral fellowships from the MENR, LNCC and ARC. This work was supported by funds from the Centre National de la Recherche Scientifique, Institut National pour la Santé et la Recherche Médicale, Institut Curie, Ligue Nationale contre le Cancer (Equipe labellisée), AICR and the European Union (RTN Program).

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Received September 24, 2001; revised December 3, 2001;
accepted December 13, 2001