Differentiation of hematopoietic cells from multipotential progenitors is regulated by multiple growth factors and cytokines. A prominent feature of these soluble factors is promotion of cell survival, in part mediated by expression of either of the anti-apoptotic proteins, BCL-2 and BCL-X\textsubscript{L}. The complex expression pattern of these frequently redundant survival factors during hematopoiesis may indicate a role in lineage determination. To investigate the latter possibility, we analyzed factor-dependent cell-Patersen (FDCP)-Mix multipotent progenitor cells in which we stably expressed BCL-2 or BCL-X\textsubscript{L}. Each factor maintained complete survival of interleukin-3 (IL-3)-deprived FDCP-Mix cells but, unexpectedly, directed FDCP-Mix cells along restricted and divergent differentiation pathways. Thus, IL-3-deprived FDCP-Mix BCL-2 cells differentiated exclusively to granulocytes and monocytes/macrophages, whereas FDCP-Mix BCL-X\textsubscript{L} cells became erythroid. FDCP-Mix BCL-2 cells grown in IL-3 were distinguished from FDCP-Mix and FDCP-Mix BCL-X\textsubscript{L} cells by a striking reduction in cellular levels of Raf-1 protein. Replacement of the BCL-2 BH4 domain with the related BCL-X\textsubscript{L} BH4 sequence resulted in a switch of FDCP-Mix BCL-2 cells to erythroid fate accompanied by persistence of Raf-1 protein expression. Moreover, enforced expression of Raf-1 redirected FDCP-Mix BCL-2 cells to an erythroid fate, and prohibited generation of myeloid cells. These results identify novel roles for BCL-2 and BCL-X\textsubscript{L} in cell fate decisions beyond cell survival. These effects are associated with differential regulation of Raf-1 expression, perhaps involving the previously identified interaction between BCL-2-BH4 and the catalytic domain of Raf-1.

Hematopoietic cells are derived from multipotential progenitors present in small numbers in the adult bone marrow. Asymmetric cell divisions generate daughter cells committed to differentiation, and subsequent cell divisions are accompanied by progressive restrictions on multilineage potential. Lineage commitment appears stochastic in single cell assays of primitive hematopoietic precursors grown in multiple growth factors (1). Certain growth factors act to increase commitment and/or expansion of specific lineages, suggesting that lineage choice is also subject to instructive signals from the microenvironment (2, 3). Owing to redundancies in signal transduction pathways from lineage-selective growth factor receptors, the processes leading to alternative lineage fates remain largely unknown. Growth factors, which also maintain cell survival, in addition provide general anti-apoptotic signals through receptors expressed on specific cell types.

Members of the BCL-2 family of apoptotic regulators have strong effects on cell survival (4–6). However, there is mounting evidence that non-lethal cell pathways are also affected by these proteins (7). For instance, BCL-2 and BCL-X\textsubscript{L} delay cell cycle re-entry. These effects can be separated from anti-apoptotic activities by mutations in the BH4 domain and, in one example, appear to involve binding of the cytoplasmic serine-threonine phosphatase, calcineurin, to the BH4 domain of BCL-2 (8, 9). This agrees with earlier reports demonstrating a block to nuclear translocation of nuclear factor of activated T cells (NFAT) with overexpression of BCL-2 in T lymphocytes (10).

BCL-2 expression influences differentiation in several cell types, including hematopoietic, neuronal, and epithelial cells (4). Mice deficient for interleukin-7 or its receptor have a block in T lymphocyte development, which can be partially restored by introduction of a BCL2\textsuperscript{transgene} (11, 12). BCL-2 rescues macrophage development in macrophage-colony stimulating factor-deficient, osteopetrotic mice (13). In addition, BCL-2 promotes regeneration and growth of severed axons in retinal ganglion cells from adult mice and accelerates neuronal differentiation in PC-12 cells (14, 15). In contrast, keratinocyte differentiation and expression of the squamous cell differentiation markers, keratin 10/11 and involucrin, are inhibited by BCL-2 (16). The effects of BCL-2 on retinal ganglion cells and keratinocytes are not attributable to the rescue of dying cells and are not reproduced by anti-apoptotic caspase inhibitors.

Adding another level of complexity, BCL-2 and BCL-X\textsubscript{L} have specific, non-homologous functions in hematopoiesis (4). BCL-2 and BCL-X\textsubscript{L} have reciprocal expression patterns in developing T and B lymphocytes and primitive human and murine hematopoietic precursor cells (17, 18). In this report, we utilize either BCL-2 or BCL-X\textsubscript{L} to maintain survival of FDCP-Mix\textsuperscript{3} progenitor cells during growth factor withdrawal. We...
demonstrate that both BCL-2 and BCL-XL enable hematopoietic differentiation in the absence of additional growth factors, but each acts along divergent and restricted pathways. Overexpression of BCL-2 supports the development of myeloid lineages and is linked to striking changes in the expression of Raf-1 kinase. In contrast, BCL-XL restricts FDCP-Mix cells to an erythroid fate. Our results provide a basis for lineage-specific cytotoxic strategies and demonstrate that BCL-2 and BCL-XL are unexpected participants in this process. Specific interactions with cytoplasmic signaling pathways may govern multiple cell fate decisions for BCL-2-related proteins with otherwise redundant survival functions.

EXPERIMENTAL PROCEDURES

Cells and Lineages of cDNAs—FDCP-Mix (clone A4) cells were grown in Iscove's modified Dulbecco's medium (Invitrogen), containing 20% horse serum (Invitrogen), and 4% rIL-3-containing supernatant (19). Cells were infected with retrovirus (MIZV; MSCV 5′ LTR-X-IRES-zeocin vector) encoding wild type or mutant Raf-1, human BCL-2, human BCL-XL, or BCL-2 BH4-mut., and selected in 0.6–0.8 mg/ml zeocin (Cayla, France) (20). Alternatively, FDCP-Mix cells were stably transduced with SFFV-Bcl2 or SFFV-BclXL plasmids by electroporation (260 volts, 960 microfarads) and selected in 0.75 mg/ml Zeocin (Invitrogen) (21). Cells were cloned by microtitering into 96-well flat-bottomed plates with 5 × 10^3 irradiated C57BL/6 spleen cells/well and 4% rIL-3 supernatant. For serial expression of two genes, FDCP-Mix cells were initially transduced with c-Raf1 retroviral vectors and subsequently transduced with BCL2 or BCLXL plasmids. Retroviral supernatants were generated bicistronically from 293T cells, along with helper plasmids. Supernatants were collected over 4 days, pooled and concentrated by ultracentrifugation. FDCP-Mix cells were spin-infected with supernatants at a multiplicity of infection of 10.1 in the presence of 4 μg/ml Polybrene.

Differentiation Assays—Cells were starved for IL-3 by washing once in phosphate-buffered saline with 5% horse serum and seeding at 3 × 10^4 cells/well in 24-well plates with Iscove's modified Dulbecco's medium containing 20% horse serum. Cytospins were performed after 40 h and stained with May-Grünwald-Giemsa (Sigma). For flow cytometry analysis of Gr-1 expression, cells were preincubated with 24G2 supernatant (Fc-block), and stained with biotinylated anti-Gr-1 or IgG2b isotype controls. Cells were subsequently stained with streptavidin-allophycocyanin (BD Pharmingen). 100,000 cells were analyzed per sample on a FACScalibur®; data were analyzed using Cell Quest software (BD Biosciences).

Antibodies—Antibodies to Raf-1 (R19120), Hsp90, BCL-2, and BCL-XL were obtained from Transduction Laboratories. Antibodies to Raf-1 (2G6.K), Gr-1, and biotinylated isotype controls were obtained from BD Pharmingen. Anti-FLAG (M2) was obtained from Sigma. Rabbit anti-mouse β-globin was obtained from ICN/Cappel and human-specific hamster anti-BCL-2 (6C8) from BD Pharmingen. Rat anti-GATA-1 was purchased from Santa Cruz Biotechnologies.

Immunoblotting—Western blots were developed with either goat anti-mouse IgG (Horse serum peroxidase (Sigma) or, for rabbit or hamster primary reagents, Protein A-horse serum peroxidase (ICN). IL-3 blasts of the indicated cell types were harvested and processed for nuclear extracts. Briefly, cells were resuspended in ice-cold hypotonic buffer, and allowed to swell for 15 min on ice. Each group was subjected to five strokes with a 27-gauge syringe, and lysis was verified by trypan blue. Nuclei were spun at 1000 rpm for 5 min in a tabletop centrifuge, and cell debris was removed. Nuclei were resuspended in hypertonic buffer for 20 min on ice. Insoluble material was removed by spinning for 5 min at 14,000 rpm. SDS sample buffer containing 2-mercaptoethanol was added to each extract, boiled for 5 min, loaded onto a 10% SDS-PAGE gel, run for 850 v-h, and processed for Western analysis.

Northern Blots—20 μg of RNA was separated on formaldehyde gels and transferred to nylon membranes by capillary transfer. Mouse cDNA probes for c-Raf1 (provided by Ulf Rapp, University of Wurzburg) and 18 S rRNA (provided by Robert Gendron, Memorial University, St. John's, Newfoundland, Canada) were labeled by random-priming (High Prime, Roche Molecular Biochemicals) with 32PdCTP (PerkinElmer Life Sciences), and purified on Sephadex G-50 columns (Amersham Biosciences).

Vectors—The BCL2BH4 mutant plasmid was kindly provided by David Huang (Walter and Eliza Hall Institute). cDNA fragments for mutant or wild type c-Raf1, BCL2, or BCLXL were amplified by PCR and ligated into the MIZV retroviral vector. The retroviral vector for bicistronic expression of BCL2/BCLXL, and green fluorescent protein (GFP) was derived from the cMMP backbone (kindly provided by the Mulligan laboratory, Harvard Medical School), into which an IRES-GFP cassette was cloned (22). Human BCL2 or BCLXL cDNAs were cloned into Xbal/BsgII sites. All constructs were verified by dyeoxy sequencing. Reagents—Geldanamycin was obtained from Sigma. ZVAD-fmk was obtained from ICN Biochemicals.

RESULTS

BCL-2 and BCL-XL Have Opposing Effects on Bipotent Cell Fate—FDCP-Mix progenitor cells were infected with MIZV retroviral vectors containing human BCL2 or BCLXL cDNAs. Zeocin (neo)-resistant clones were derived from single cells transferred to 96-well plates by micromanipulation. Multiple clones with high expression of human BCL-2 or BCL-XL protein by immunoblotting were selected for further study.

Non-transduced FDCP-Mix cells, and FDCP-Mix zeo, FDCP-Mix BCL-2, and FDCP-Mix BCL-XL clones had similar growth characteristics and maintained undifferentiated blast morphology in the presence of IL-3 (IL-3 blasts; Fig. 1A, B, and E). IL-3 withdrawal from FDCP-Mix BCL-2 or FDCP-Mix BCL-XL clones prevented cell growth, but viability remained >95% for at least 48 h. In contrast, both FDCP-Mix zeo and non-transduced FDCP-Mix cells underwent rapid death upon IL-3 withdrawal (>90% of cells were trypan blue-positive at 24 h).

Cytospins of FDCP-Mix BCL-2 clones were performed after 40 h of IL-3 withdrawal and examined by May-Grunwald-Giemsa staining. A complete shift in morphology to granulocytes, monocytes, and macrophages was observed. The ratio of granulocytes to monocytes/macrophages among different FDCP-Mix BCL-2 clones varied from 50:50 to 30:70. In 20 different FDCP-Mix BCL-2 clones examined, fewer than 2% of cells with erythroid morphology were observed. FDCP-Mix BCL-XL clones were examined similarly. In striking contrast to the results for BCL-2, BCL-XL-expressing cells uniformly gave rise to erythroblasts upon IL-3 deprivation, with virtually no granulocytes or monocytes observed. This result was also repeated in 20 separate clones.

Among a larger group of 40 clonal lines of each type, 35/40 FDCP-Mix BCL-2 and 39/40 FDCP-Mix BCL-XL clones produced the same pattern of restricted myeloid and erythroid differentiation, respectively, upon IL-3 withdrawal (Fig. 1, C and F). A small number of phenotypic variants were observed in each of the two groups of transduced FDCP-Mix clones. These clones also had restricted differentiation but with the opposite pattern to the majority of clones. Thus, 5/40 FDCP-Mix BCL-2 and 1/40 FDCP-Mix BCL-XL clones produced restricted erythroid and myeloid phenotypes, respectively, after IL-3 withdrawal (Fig. 1, D and G). Notably, expression levels of human BCL-2 in the FDCP-Mix clones did not correlate with specific lineage fate (see Fig. 3A).

To address the possibility that the restricted differentiation phenotypes we observed were a result of the cloning process, we generated bicistronic BCL2 and BCLXL-expressing retroviral vectors containing an internal ribosomal entry site (IRES) upstream of the GFP marker gene. FDCP-Mix cells were sorted for GFP expression by flow cytometry at 72 h after transduction (Fig. 1H). We found that resuspending the cells in 0.2% rIL-3 medium (20-fold dilution) slowed the death of control cells, such that >60% of cells remained viable at 40 h. Sorted GFP-positive cells were replated in 0.2% IL-3 and examined for differentiation morphology after 40 h. Control FDCP-Mix IRES-GFP cells retained a primitive cell morphology under these conditions (Fig. 1J). Cells transduced with the BCL2-GFP vector differentiated into myeloid lineage cells in 0.2% IL-3; in contrast, IL-3-deprived cells expressing BCLXL-GFP contained exclusively erythroblasts (Fig. 1, J and K).

To quantify the restricted differentiation in the FDCP-Mix...
BCL-2 and FDCP-Mix BCL-X<sub>L</sub> clones, we analyzed for lineage-specific markers. FDCP-Mix BCL-2 clones acquired the granulocytic marker, Gr-1, upon IL-3 withdrawal, whereas FDCP-Mix BCL-X<sub>L</sub> cells remained Gr-1-negative by flow cytometry (Fig. 2A). Erythroid differentiation was evaluated by expression of the erythropoietin receptor, by reverse transcriptase (RT)-PCR analysis, and β-globin, by immunoblotting. RT-PCR amplification of EpoR mRNA was detected in IL-3-deprived FDCP-Mix BCL-X<sub>L</sub> clones (data not shown). FDCP-Mix BCL-X<sub>L</sub> clones produced high levels of mouse β-globin in the absence of IL-3, with little to no β-globin expressed in FDCP-Mix BCL-2 clones (Fig. 2B). Similarly, FDCP-Mix BCL-X<sub>L</sub>, but not FDCP-Mix BCL-2 clones, expressed the erythroid-specific transcription factor GATA-1 (Fig. 2C). These results confirm the morphologic assessment of restricted myeloid and erythroid differentiation. Thus, BCL-2 expression dictates myeloid lineage choice, and BCL-X<sub>L</sub> expression specifies erythroid cell fate, under circumstances in which selective cell survival appears to play no role.

**Raf-1 Protein Expression in FDCP-Mix IL-3 Blasts Predicts Differentiation Pattern of BCL-2/BCL-X<sub>L</sub> Expression**—In addition to heterodimer associations with pro-apoptotic family members, BCL-2 physically interacts with several cytoplasmic proteins, including calcineurin, Raf-1 kinase, Bag-1, and p23-R-Ras (6). In a survey of these proteins, we observed differences in Raf-1 expression between FDCP-Mix BCL-2 and FDCP-Mix BCL-X<sub>L</sub> clones. Raf-1 protein was undetectable by immunoblotting of total cell lysates from all FDCP-Mix BCL-2 clones that formed myeloid cells (denoted FDCP-Mix BCL-2-myeloid). Importantly, the loss of Raf-1 expression was observed in FDCP-Mix BCL-2-myeloid clones grown in high concentrations of IL-3 and thus preceded differentiation. In contrast, Raf-1 protein was consistently observed in FDCP-Mix BCL-X<sub>L</sub>-erythroid clones at levels comparable to non-transduced and FDCP-Mix neo controls (Fig. 3, A and B). We obtained an identical result with two different Raf-1-specific antibodies (Fig. 3A).

The rare BCL-2 and BCL-X<sub>L</sub> clones with variant differentiation phenotypes gave discordant results. We failed to detect Raf-1 in the single FDCP-Mix BCL-X<sub>L</sub>-myeloid clone isolated, but each of the five FDCP-Mix BCL-2-erythroid clones consistently expressed Raf-1 protein (Fig. 3, A and B). Thus, presence or absence of Raf-1 protein in undifferentiated IL-3 blasts predicted with complete accuracy the differentiation phenotype for that clone upon IL-3 withdrawal. The influence of Raf-1 expression on cell differentiation takes precedence over the effects of BCL-2 and BCL-X<sub>L</sub>, suggesting that Raf-1 acts downstream of BCL-2 and BCL-X<sub>L</sub>. However, differences in Raf-1 expression are unrelated to the anti-apoptotic functions of BCL-2 and BCL-X<sub>L</sub>, as IL-3-independent survival of both sets of FDCP-Mix BCL-2 and FDCP-Mix BCL-X<sub>L</sub> clones exceeded 95% at 40 h.

The Raf family of serine/threonine kinases includes two additional members, A-Raf and B-Raf. B-Raf is selectively expressed in nervous tissue, whereas Raf-1 and A-Raf are more widely expressed (23). We examined A-Raf expression by immunoblotting. The 64-kDa A-Raf protein was expressed at comparable levels in IL-3 blasts of FDCP-Mix BCL-2, FDCP-Mix BCL-X<sub>L</sub>, and FDCP-Mix neo cells, with down-regulation in IL-3-deprived cells (Fig. 3C). Thus, the loss of Raf-1 expression in FDCP-Mix BCL-2-myeloid cells does not reflect a general suppression of protein expression but appears to be specific for Raf-1, even among closely related family members.

Northern blots of total cytoplasmic RNA demonstrated equivalent expression of c-raf1 RNA in FDCP-Mix BCL-2, FDCP-Mix BCL-X<sub>L</sub>, and FDCP-Mix neo control cells (Fig. 4). Moreover, c-raf1 RNA levels were comparable in FDCP-Mix BCL-2-myeloid and FDCP-Mix BCL-2-erythroid clones and, therefore, independent of specific differentiation phenotype. These results indicate a post-transcriptional mechanism for the decrease in Raf-1 protein in the presence of BCL-2.

**Raf-1 Down-regulation Requires the BCL-2 BH4 Domain**—The conserved BCL-2 homology 4 (BH4) domains are NH<sub>2</sub>-proximal regions of approximately 20 amino acids each (Fig. 5E). The BCL-2 and BCL-X<sub>L</sub> BH4 regions differ in the relative
BCL-2-BH4 Domains Regulate Raf-1 Expression and Cell Fate

Fig. 2. Lineage marker analysis of representative FDCP-Mix BCL-2 or FDCP-Mix BCL-XL clone. A, cells were IL-3-deprived for 40 h and stained with anti-Gr-1 (open histograms) or IgG2b isotype control (shaded histogram). Gr-1 staining was reproduced in 10/10 FDCP-Mix BCL-2-myeloid clones tested. B, immunoblot analysis for mouse β-globin; 2 × 10⁶ cell equivalents per lane of whole cell lysates. Control Buffy coat (−) control, human peripheral blood. C, immunoblot analysis for GATA-1; nuclear extracts from 5 × 10⁶ cells (lanes 3 and 5), 1.5 × 10⁷ cells (lane 2), and 2.5 × 10⁷ cells (lanes 1 and 4).

spacing of charged and polar residues. Although the BCL-2-BH4 domain has been found to associate with Raf-1, Bag-1, and calcineurin, protein-protein interactions involving the BCL-XL-BH4 domain have not been described in similar detail (6, 9, 10, 24). To determine if the BH4 domains of BCL-2 and BCL-XL are involved in their specific effects on cell fate and Raf-1 protein levels, we tested a chimeric BCL-2 protein in which the endogenous BH4 domain is replaced by the homologous sequence from BCL-XL. This BCL-2 BH4βδελαε mutant has full survival function (25). A corresponding BCL-XL construct bearing the BH4 domain sequence lacked survival function and could not be further evaluated.

FDCP-Mix cells were stably infected with retrovirus containing BCL2 BH4βδελεαε. In contrast to the restricted myeloid differentiation seen with BCL-2, FDCP-Mix BCL-2 BH4βδελεαε clones formed uniformly erythroid cells when deprived of IL-3 (Fig. 5, B and C). Furthermore, immunoblots revealed that expression of endogenous Raf-1 was restored in BCL-2 BH4βδελεαε clones in the presence of IL-3 (Fig. 5A).

Cell survival was maintained at high levels over 5 days for IL-3-deprived FDCP-Mix BCL-2 BH4βδελεαε, FDCP-Mix BCL-2, and FDCP-Mix BCL-XL clones, indicating that the specific differentiation effects of the anti-apoptotic proteins are loosely coupled to survival functions (Fig. 5D). The ability of BCL-2 to slow cell cycle re-entry can also be dissociated from its survival function by mutations within the BH4 region (8). A role for the BCL-2-BH4 domain in down-regulating Raf-1 protein expression is consistent with this helical domain as a site of protein-protein interactions and supports the hypothesis that Raf-1 is an effector of BCL-2 differentiation activity.

Expression of Raf-1 Converts FDCP-Mix BCL-2 Cells to Erythroid Fate—To test whether Raf-1 operates as a switch for lineage choice in FDCP-Mix cells, we generated FDCP-Mix cell lines co-expressing Raf-1 and BCL-2 (Fig. 6A).

The introduction of Raf-1 redirected FDCP-Mix BCL-2 cells to an exclusively erythroid fate in 20/20 individual clones analyzed (Fig. 6B). FDCP-Mix Raf-1-BCL-2 cells also expressed GATA-1, in contrast to cells transduced with BCL2 alone (Fig. 6C). Co-expression of Raf-1 and BCL-XL did not alter the predominant erythroid phenotype associated with FDCP-Mix BCL-XL cells (Fig. 6, D–F). Transduction of c-raf1 by itself did not confer IL-3-independent survival or induce differentiation of FDCP-Mix cells (data not shown). However, the addition of zVAD-fmk, a pan-caspase inhibitor, to FDCP-Mix Raf-1 cells allowed early erythroid differentiation following IL-3 deprivation (Fig. 6H). In contrast to the accumulation of differentiated cells observed with BCL-2 or BCL-XL, IL-3-deprived FDCP-Mix cells maintained an undifferentiated appearance in the presence of zVAD-fmk (Fig. 6O).

Co-expression of a constitutively active, truncated Raf-1, including only the catalytic CR3 domain, CAT-Raf (26), also switched FDCP-Mix BCL-2 cells to an erythroid fate following IL-3 withdrawal (data not shown). Surprisingly, a kinase-defective mutant of CAT-Raf, CAT-Raf-K375M, gave identical results (Fig. 7A). Erythroid morphologies were accompanied by expression of mouse β-globin (Fig. 7B).

We demonstrated expression of the epitope-tagged 36-kDa CAT-Raf and CAT-Raf-K375M proteins using a FLAG-specific antibody (Fig. 7C and data not shown). Eight individual FDCP-Mix CAT-Raf-K375M/BCL-2 clones are shown in lanes 2–9. We also observed re-expression of 74-kDa Raf-1 using anti-Raf-1 antibodies (Fig. 7C). Levels of endogenous Raf-1 protein in the CAT-Raf-BCL-2-transduced clonal lines were generally higher than in non-transduced FDCP-Mix cells. One FDCP-Mix CAT-
BCL-2-BH4 Domains Regulate Raf-1 Expression and Cell Fate

Fairbairn et al. (30) previously reported that IL-3-dependent FDCP-Mix hematopoietic progenitor cells transduced with BCL-2 underwent spontaneous multilineage differentiation following removal of IL-3. Rather than conventional stochastic or inductive differentiation models, these experiments indicated that, in the presence of sufficient intrinsic survival factors, the requirement for specific extrinsic differentiation signals is bypassed.

In the present study, we used a similar approach to determine if BCL-2 and BCL-XL shared an ability to support hematopoietic differentiation. BCL-2 has been suggested to act passively on differentiation outcomes by maintaining cell survival during a vulnerable period. However, the side-by-side comparisons shown here suggest a more complex relationship between BCL-2-related survival proteins and differentiation, because BCL-2 and BCL-XL, as well as the BCL-2 BH4/chima, all maintain survival of IL-3-deprived FDCP-Mix cells, while directing alternative lineage fates. The reproducibility of the BCL-XL-erythroid and BCL-2-myeloid associations across multiple independent clones argues against random, mutational events that could restrict multipotentiality. Moreover, selection and expansion of clones were performed in the presence of IL-3, such that the eventual lineage choices remained latent. Finally, transient assays using a bicistronic vector to enable sorting of GFP-positive, transduced cells demonstrated identical phenotypes.

Our results extend previous views of lineage determination by demonstrating that the intrinsic survival factors, BCL-XL and BCL-2, are not necessarily passive participants but can also direct (specify) differentiation fates. Because hematopoietic growth factors induce the expression of specific BCL-2

**Fig. 3. Differentiation phenotype correlates with expression of Raf-1.** Analysis of Raf-1 and A-Raf in FDCP-Mix BCL-2 and FDCP-Mix BCL-XL clones. Individual clonal lines are designated by number following expressed gene. Morphology of FDCP-Mix clones in absence of IL-3 is indicated. A, immunoblots probed with (i) R19120-anti-Raf-1; (ii) clone UR1026K-anti-Raf-1; (iii) anti-human BCL-2 or anti-BCL-XL. Positive control for Raf-1 and BCL-2 shown by (+). FDCP-Mix BCL-XL clones 56 and 22 (lanes 11 and 12) yield erythroid phenotypes. Inset, overexposure of lanes 7–10. B, FDCP-Mix BCL-XL (erythroid) clones examined for expression of Raf-1 (R19120) or BCL-XL. C, immunoblot for p64 A-Raf or BCL-2 in presence of IL-3 or after 40 h of IL-3 withdrawal. 1 × 10⁶ cell equivalents per lane of whole cell lysates.

Raf-K375M/BCL-2 clonal cell line, #33 (lane 7), which produced mixed erythroid and myeloid lineages, contained notably lower expression of the Raf-1 CR3 domain mutant and lacked detectable endogenous Raf-1 protein. CAT-Raf should not interfere with Ras-dependent activation of endogenous Raf-1, due to the absence of the NH₂-terminal Ras-binding domain.

These results suggest that expression of the Raf-1 CR3 domain inhibits the post-transcriptional down-regulation of endogenous Raf-1 associated with BCL-2 expression, thereby preserving Raf-1 kinase levels. Because both kinase-active and inactive CAT-Raf proteins rescued expression of endogenous Raf-1 protein, Raf-1 kinase activity is dispensable for this effect. Previous work demonstrated an interaction of the Raf-1 CR3 domain with the BCL-2 BH4 domain (24). Raf-1 CR3 domains may act by interfering with protein-protein interactions between Raf-1 and another factor, possibly BCL-2, independent of associated kinase activity.

As a further test of the role for Raf-1, we determined the effect of reducing Raf-1 expression in FDCP-Mix BCL-XL cells.
family members (31, 32), survival and differentiation functions could converge in a single multitasking protein as a striking example of cellular economy.

In screening for expression of proteins previously reported to associate with BCL-2 or BCL-XL, we discovered that Raf-1 protein could not be detected in immunoblots of FDCP-Mix BCL-2 cell lysates. Only 5/40 FDCP-Mix BCL-2 clones had measurable Raf-1 protein, and remarkably, each gave rise to erythroid cells following removal of IL-3. Raf-1 protein levels in FDCP-Mix BCL-XL clones were comparable to control cells. FDCP-Mix BCL-XL and FDCP-Mix BCL-2 clones expressed similar levels of c-raf1 mRNA, indicating a post-transcriptional level of regulation. Raf-1 protein was undetectable in only 1 out of 40 independent FDCP-Mix BCL-XL clones, and this clone reproducibly differentiated to myeloid cells upon IL-3 withdrawal. Thus, in every instance, where Raf-1 protein is observed, erythroid differentiation ensues and absence of Raf-1 is accompanied by myeloid differentiation.

The importance of Raf-1 in FDCP-Mix cell fate was demonstrated by showing that introduction of c-raf1 into FDCP-Mix BCL-2 cells switched the differentiated phenotype from myeloid to erythroid upon IL-3 withdrawal. This result makes evident an epistatic relationship between Raf-1 and BCL-2, which was previously suggested by analysis of FDCP-Mix BCL-2 clones with variant differentiation phenotypes. In this context, it is notable that constitutive expression of Raf-1 by itself, when coupled with the neutral inhibitor of apoptosis, zVAD-fmk, initiates erythroid differentiation once IL-3 is removed. Conversely, accelerated turnover of Raf-1 protein in geldanamycin-treated FDCP-Mix-BCL-XL cells is associated with redirection to myeloid differentiation.

Both constitutively active, truncated Raf-1 (CAT-Raf) and
BCL-2-BH4 Domains Regulate Raf-1 Expression and Cell Fate

CAT-Raf with an active site mutation to disable kinase activity produced a switch of FDCP-Mix BCL-2 differentiation from myeloid to erythroid. Thus, the kinase activity of the introduced Raf-1 appears to be irrelevant for production of erythroid cells. However, both CR3-only c-raf1 constructs led to restoration of endogenous Raf-1 protein levels in BCL-2-expressing FDCP-Mix cells. This result suggests that the disappearance of endogenous Raf-1 protein requires the COOH-terminal CR3 domain, and furthermore, that CR3-only mutant proteins compete with endogenous Raf-1 for binding to another factor.

BCL-2, via its Raf-1-binding BH4 domain, is one candidate for this factor. We show that a BCL-2 mutant with its BH4 domain replaced by its counterpart in BCL-XL does not lead to loss of Raf-1 expression and that FDCP-Mix cells expressing this BCL-2 BH4BLX chimera are directed to an erythroid fate after IL-3 washout. This identifies BH4 as a structural domain of BCL-2 that regulates both Raf-1 expression and lineage differentiation (42). Fetal liver hematopoietic cells die prematurely in vivo, with levels increasing 50-fold during terminal erythroid differentiation (42). Fetal liver hematopoietic cells die prematurely in BCLXL-deficient mice, and conditional deletion of BCLXL causes anemia with increased death of late erythroid precursors (43, 44). BCLXL-deficient ES cells do not generate mature, viable definitive erythroid cells in vitro and, moreover, fail to contribute to circulating erythrocytes in adult chimeric mice (45).

BCL-2 is expressed primarily in the B and T lymphoid compartments and in early hematopoietic precursors. Expression of a vav-BCL2 transgene in hematopoietic cells produced large increases in numbers of peripheral blood lymphocytes and monocytes, but not erythrocytes, and was accompanied by a 2-fold reduction in nucleated erythroblasts in the bone marrow (46). Two groups have reported that Raf-1 is not expressed in primary neutrophils (47, 48). We confirmed the absence of Raf-1 in CD15⁺ human neutrophil lysates (data not shown).
whereas Raf-1 was readily detected by immunoprecipitation in lysates of CD15+ cells (primarily lymphocytes, monocytes, and eosinophils). Similarly, granulocytic differentiation of HL60 promyeloid cell line is accompanied by a striking loss of Raf-1 protein (48). Our observations in FDCP-Mix BCL-2 cell lines implicating BCL-2, BCL-XL, and BCL-2 BH4Bcl-x into primary both erythroid and myeloid-specific values of the parameter cell population. At either extreme, represented by high expression of BCL-2 and BCL-XL, and subsequent effects on Raf-1, cell differentiation becomes skewed toward myeloid and erythroid cell fates, respectively. This model might explain the variable effects of manipulating BCL-2 and BCL-XL expression on hematopoietic lineage determination in published in vivo and in vitro models. At intermediate levels of BCL-2 or BCL-XL, both erythroid and myeloid-specifying values of the parameter would be represented and bi-phenotypic patterns of differentiation would be observed. Direct comparisons of the effects of introducing BCL-2, BCL-XL, and BCL-2 BH4Bcl-x into primary multipotent hematopoietic progenitors may offer further insights into normal mechanisms of lineage restriction during hematopoiesis (49).

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