Activated abl Oncogenes and Apoptosis: Differing Responses of Transformed Myeloid Progenitor Cell Lines

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Activation of the c-abl protooncogene occurs during the generation of both the Abelson murine leukemia virus and the bcrabl fusion gene. To further dissect the biological properties of these proteins, we studied their effect on apoptosis. Using dimethyl sulfoxide (DMSO) to induce apoptosis in the murine myeloid progenitor cell line 32Dc13, we examined the effect of expression of both v-abl and bcrabl transgenes on apoptosis. v-abl expressing 32Dc13 cells are sensitive to apoptosis induction, similar to parental 32Dc13 cells. In contrast, bcrabl expressing 32Dc13 cells are protected from the apoptotic stimulus resulting from DMSO exposure. Analyzing the expression patterns for Bcl-2 and Bax, two proteins known to modulate the apoptotic response, we found a downregulation of Bcl-2 and enhanced expression of Bax in 32Dc13 cells. In 32Dc13/v-abl cells, Bcl-2 expression remained constant while Bax was upregulated, whereas in 32Dc13 cells expressing bcrabl, there was continuous expression of Bcl-2 at a level greater than observed in v-abl transformed cells. Taken together, our data demonstrate that although both activated abl gene products promote overlapping effects of some biological responses (ie, factor-independent proliferation) they diverge in their effect on apoptotic signaling pathways.

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THE PROTOONCOGENE c-abl is a nonreceptor tyrosine kinase whose biological effect is mediated through its tyrosine kinase activity. This activity is tightly controlled, resulting in the lack of detectable autophosphorylated c-Ab1 protein in vivo. In contrast, the activated oncogenic forms of c-abl, ie, v-abl and bcrabl, encode gene products that are autophosphorylated on tyrosine in vivo. This is indicative of the deregulation of their kinase activity, a critical step in activating their oncogenic potential. v-abl and bcrabl share numerous biochemical and biological properties, including a structurally modified 5' end, enhanced tyrosine kinase activity and the acquisition of transforming and tumorigenic properties. Although the 5'-ends of v-abl and bcrabl vary, both genes retain the c-abl sequences that encode for the SH2 domain, for tyrosine kinase activity, and, within the 3' terminus separate domains, for both DNA and actin binding. While structurally similar to c-abl, biologically the activated v-abl and bcrabl homologues do not resemble it. Differences in subcellular localization, growth effects, and phosphotyrosine modification of the encoded proteins are dramatic. Whereas c-Ab1 protein is primarily localized to the nucleus, both activated v-Ab1 and BcrAbl proteins are cytoplasmic, though retaining the c-abl nuclear localization motif. Overexpression of c-abl has been reported to inhibit growth in NIH/3T3 fibroblasts, whereas expression of v-abl results in foci formation. Finally, when lethally irradiated mice are reconstituted with bone marrow cells infected with either v-abl or bcrabl expressing retroviruses, a chronic myelogenous-like leukemia is induced.

Expression of both activated Abl oncoproteins induces many common phenotypes in cells. For example, factor-dependent hematopoietic cell-lines expressing the activated Abl oncoproteins are rendered cytokine-independent. When cytokines are withdrawn from factor-dependent cells, activation of apoptosis ensues, however, expression of either v-abl or bcrabl blocks this apoptotic response and rather than die, the cells continue to proliferate. 32Dc13 myeloid precursor cells will differentiate to granulocytes when cultured in the presence of granulocyte colony stimulating factor (G-CSF), however, expression of either v-abl or bcrabl blocks G-CSF-induced differentiation.

The effects of v-Ab1 and BcrAbl oncoprotein expression are not, however, totally synonymous. One biological assay that distinguishes between these two gene products is focus formation using NIH/3T3 cells. v-abl expression induces transformed foci, while bcrabl expression does not. These findings suggest that although v-Ab1 and BcrAbl proteins affect common pathways, distinct interactions characteristic to the individual oncoproteins also occur. Some of these unique characteristics of v-Ab1 versus BcrAbl may be attributed to differences in their subcellular localization. The v-Ab1 protein exists as a Gag-Ab1 fusion protein, and the presence of both the Gag polypeptide and the gag encoded myristylation motif localizes the protein to the membrane. In contrast, BcrAbl protein contains two separate actin binding domains, in both c-Ab1 and c-Bcr, resulting in the association of BcrAbl protein with actin filaments.

Towards further dissecting the role of these oncogenes in tumorigenicity, we sought to assess how cellular responses to different apoptotic-inducers were affected by v-Ab1 as opposed to BcrAbl oncoprotein expression. To do so, v-abl and bcrabl transgenes were introduced into the factor-dependent murine myeloid progenitor cell-line 32Dc13. This is an immortalized, diploid, interleukin-3 (IL-3)-dependent clonal cell line derived from normal mouse bone marrow that is competent to terminally differentiate into granulocytes in the presence of G-CSF. Previously, either v-abl or bcrabl expression in these cells was shown to abrogate IL-3 dependence, to suppress the induction of apoptosis that ensues following cytokine depletion, and to block the induction of terminal granulocytic differentiation by G-
CSF.\textsuperscript{[10]} However, whether v-Abl and BcrAbl proteins may differ in their effect on cells exposed to apoptotic stimuli was not determined. Also, what effect of these distinct abl oncogenes may have on bcl-2 and bax expression is uncertain; two gene products that have been identified as critical components in promoting cell survival and cell death, respectively.\textsuperscript{[18,19]}

To answer these questions, 32Dc13 cell lines that express v-abl and bcrabl have been established and their response to the induction of apoptosis by dimethyl sulfoxide (DMSO) has been examined. It is reported that (1) ectopic expression of v-abl did not block the DMSO-induced apoptosis of 32Dc13 cells, whereas bcrabl expression inhibited apoptosis induction by DMSO (2) both IL-3 and overexpression of bcl-2 increase the survival of both parental 32Dc13 cells and 32Dc13/v-Abl cells during DMSO treatment, but cannot block the induction of apoptosis, and (3) although the responsiveness to DMSO-induced apoptosis among these cell lines correlated with the levels of endogenous Bcl-2 and Bax protein expression, the inability of bcl-2 overexpression to block apoptosis when expressed at levels similar to 32Dc13/BcrAbl cells indicates that other proteins play a critical role in cell survival. This diverse response of 32Dc13 cells ectopically expressing v-Abl and BcrAbl oncoproteins to DMSO-induced apoptosis is in contrast to the common ability of these oncoproteins to inhibit apoptosis in 32Dc13 cells following IL-3 withdrawal. These results indicate that the different oncogenic forms of the c-abl protooncogene differentially effect apoptotic pathways, thereby providing a model system to further dissect their distinct roles in oncogenicity.

MATERIALS AND METHODS

Cell culture and DNA transfection. The murine myeloid progenitor cell line 32Dc13\textsuperscript{[12,17]} was cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Biowhittaker) and 10% WEHI-3B cell\textsuperscript{[28]} conditioned medium as a source of IL-3, in a 37°C incubator in a humidified atmosphere with 5% CO\textsubscript{2}. v-abl encoding the P160 kD protein (gift of Dr S. Goff, Columbia University, New York, NY) and bcrabl\textsuperscript{[46]} were subcloned into the EcoRI site of a modified pMV7\textsuperscript{70} retroviral vector (gift of Dr I.B. Weinstein, Columbia University, New York, NY) that had the neo expression cassette deleted (data not shown). pMV7 recombinant constructs containing the various cDNA inserts were linearized by digestion with Pvu I, and 20 µg of linearized plasmid DNA was electroporated into 4 x 10\textsuperscript{7} 32Dc13 cells using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) with 300 V and 960 µF. Individual transfected clones were selected by plating cells in IMDM lacking IL-3, but containing 20% FBS and made 0.3% with Noble agarose (Difco, Detroit MI). To generate 32Dc13 cell lines expressing human Bcl-2, pZip-bcl-2 plasmid was linearized with Nde I before electroporation. pZip-neo vector alone was introduced into cells in an identical manner and served as a negative control. Transfectants were selected following plating into soft agar with the addition of 1 mg/mL G418 (Life Technologies, Grand Island, NY). Five independent clones of 32Dc13/BcrAbl and three each of 32Dc13/v-Abl were found to be similar to the clones presented in this study. Individual clonally selected 32Dc13/neo cell lines were found to be indistinguishable from the parental cells in their response to both DMSO and IL-3 withdrawal.

DMSO treatment. DMSO American Chemical Society (ACS) grade (Sigma, St Louis, MO) was added to culture medium to a final concentration of 1.5%. Cells were seeded at a concentration of 4 x 10\textsuperscript{3} cells/mL for the various viability experiments. Viability was determined by trypan blue dye exclusion and counting with a hemocytometer. A minimum of duplicate independent determinations was used to derive the means that are presented graphically, with standard deviations up to 15% (ie, 90% viability ± 1.5%).

Protein analysis. The expression of enzymatically active P160 v-Abl and P210 BcrAbl was assayed using the immune complex kinase assay as described previously by Shore et al\textsuperscript{[14]} using an anti-Abl monoclonal antibody 8E9 (gift of Drs R. Arlinghaus, M.D. Anderson, Houston, TX and J.Y.J. Wang, University of California at San Diego, San Diego, CA). Expression of murine Bax p21, murine Bcl-2 p26, and transfected hu-Bcl-2 was confirmed by Western blotting. For Western blotting experiments, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) plus the protease inhibitors 2 µg/mL leupeptin, 2 µg/mL aprotinin, 2 µg/mL pepstatin, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)) and protein concentrations were determined (Bio-Rad). Equivalent amounts of cell lysates (100 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were transferred electrophoretically (LBK Transblot, Upssala Sweden) to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The blots were probed with either an anti-Bcl2 monoclonal antibody (MoAb) specific for hu-Bcl-2 (Oncogene Science, Uniondale, NY), an anti-MoAb-Bcl-2 MoAb, or an anti-Bax polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibodies (Vector Labs, Burlingame, CA) and enhanced chemiluminescence (NEG/DuoPoint, Boston, MA).

DNA fragmentation analysis. A total of 1 to 2 x 10\textsuperscript{6} cells were washed in phosphate-buffered saline (PBS) and resuspended in 300 µL of DNA extraction buffer containing 10 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 5 mmol/L EDTA, 0.5% SDS, and 500 µg/mL Proteinase K (Boehringer Mannheim, Indianapolis, IN). After incubation overnight at 37°C, the cell lysates were extracted sequentially with an equal volume of Tris buffer-saturated phenol, Tris buffer saturated phenol-chloroform and chloroform-isooamyl alcohol and then the DNA was precipitated. The DNA was resuspended in 10 mmol/L Tris pH 7.5, 1 mmol/L EDTA (TE). RNase A (1 mg/mL) and RNase T1 (500 U/mL; Life Technologies) were added and incubated for 1 hour at 37°C, then extracted two times and precipitated. The DNA was resuspended in TE buffer and the concentration was determined by optical density (OD) at 260 nm. A total of 50 µg of each DNA sample was loaded on a 2% agarose gel containing 0.1 µg ethidium bromide/mL, electrophoresed using 1 x TBE (0.05 mol/L Tris base, 0.05 mol/L Boric acid, 1 mmol/L EDTA) as the running buffer and visualized with ultraviolet (UV) illumination.

RESULTS

Generation of 32Dc13 cell lines expressing the activated Abl oncoproteins P160 v-Abl and P210 BcrAbl. To assess the effect of different Abl oncoproteins on apoptosis, we introduced an expression vector containing either the P160 v-abl or P210 bcrabl cDNA insert into 32Dc13 cells by electroporation. Clonal cell lines expressing the different abl oncogenes were isolated by plating the transfected cells in growth medium containing 0.3% agar, but devoid of IL-3, and selecting single colonies. Transfected 32Dc13 cells expressed either p160 v-Abl or p210 BcrAbl protein that was enzymatically active, as determined by an immune complex kinase analysis (data not shown). It was also observed that expanded cell populations of 32Dc13/v-Abl or 32Dc13/
BcrAbl clones were observed to proliferate in medium lacking IL-3 and failed to differentiate in response to the addition of G-CSF (Fig 1 and data not shown), indicating that oncogenic transformation of 32Dc13 cells by either Abl oncoprotein abrogate (1) cytokine-dependent proliferation, (2) apoptosis following cytokine withdrawal, and (3) cytokine-induced differentiation.

**Induction of apoptosis by DMSO in 32Dc13 cells and 32Dc13 cells expressing v-abl and bcrabl.** DMSO is known to induce differentiation in some hematopoietic cell lines, including a subclone of 32D cells. However, the clonal isolate, 32Dc13 has been reported to be refractory to the induction of granulocytic differentiation by this agent. We observed that when parental 32Dc13 cells were cultured in the presence of IL-3 and 1.5% DMSO, there was a decline in cell viability, although not as rapid as that resulting following withdrawal of IL-3 (Fig 1A). To determine if this loss of viability, resulting from exposure to DMSO, was due to programmed cell death, we used endonucleolytic cleavage of the genomic DNA into oligonucleosome ladders as a diagnostic indicator of apoptosis. DNA ladders were detected 6 hours after the addition of DMSO in 32Dc13 cells cultured in the presence of IL-3, and the intensity of the DNA fragments increased over the exposure period (Fig 1D). Thus, rather than inducing differentiation, DMSO exposure results in the apoptotic death of 32Dc13 cells. To further evaluate DMSO in the induction of apoptosis, 32Dc13 cells were cultured in medium containing 1.5% DMSO, but devoid of IL-3. As shown in Fig 1A, an additive effect was consistently observed; the loss of 32Dc13 cell viability occurred more...
rapidly than following either DMSO exposure only or withdrawal of IL-3.

As shown in Fig 1B, 32Dc13 cells expressing either v-abl or bcrabl were equally capable of proliferation in the absence of IL-3, in contrast to the parental 32Dc13 cell line, which rapidly lost viability when IL-3 was withdrawn. Thus, the expression of either activated Abl oncoprotein substituted for the proliferative and survival properties provided by IL-3 to 32Dc13 cells and efficiently blocked induction of apoptosis following its removal. To determine if activated Abl protein expression in the transgenic cell lines also was capable of blocking the induction of apoptosis by DMSO, we treated the cell lines with 1.5% DMSO. Viability assays were performed both in the presence and absence of IL-3, to compare their response to the parental 32Dc13 cell line. Exposure of 32Dc13 cells expressing v-abl to 1.5% DMSO in the presence of IL-3, resulted in a loss of viability that was similar to that observed for the parental cells over a 4-day span (Fig 1C). However, at day 6 of exposure, the viability of the 32Dc13/v-Ab1 cells was greater than the parental cells (43% ± 15%), indicating that v-Ab1 can promote some survival on induction of apoptosis by DMSO. To verify that DMSO treatment was indeed inducing apoptosis in the 32Dc13/v-Ab1 cells, DNA was isolated and oligonucleosome ladders were readily apparent beginning 6 hours after DMSO treatment (Fig 1D).

We next cultured the 32Dc13/v-Ab1 cells in medium devoid of IL-3 but containing 1.5% DMSO to determine this effect on apoptosis induction. Figure 1C shows that 32Dc13/v-Ab1 cells cultured in medium devoid of IL-3, but containing 1.5% DMSO, lost viability at a faster rate throughout the entire exposure period compared with 32Dc13/v-Ab1 cells cultured in the presence of both IL-3 and DMSO. However, the loss in viability was less severe than that observed for 32Dc13 parental cells treated in an identical manner (Fig 1C). Thus, in both the parental and v-Ab1 expressing 32Dc13 cells, IL-3 promotes survival in the presence of DMSO, despite their varied requirements for IL-3. In sharp contrast, 32Dc13/BcrAb1 cells cultured in the presence of both 1.5% DMSO and IL-3 remained viable (Fig 1C) and retained the integrity of their genome (Fig 1D). Similarly, the survival of these cells was unaffected on culturing in medium containing DMSO, but devoid of IL-3 (Fig 1C). The biological effect of these two Abl oncoproteins can, thus, be distinguished by their ability to block the induction of apoptosis by DMSO.

Taken together these observations show (1) that DMSO induces apoptosis in both parental 32Dc13 and 32Dc13 cells expressing v-Ab1 in the presence of IL-3, indicating that DMSO-induced apoptosis is distinct from apoptosis induced following IL-3 withdrawal, (2) even though v-Ab1 expression abrogates the dependence of 32Dc13 cells on IL-3 for survival and proliferation, following exposure to DMSO, IL-3 does promote cell survival in both the parental and the 32Dc13/v-Ab1 cells, and (3) DMSO induces apoptosis in both parental 32Dc13 and 32Dc13/v-Ab1 cells, while expression of bcrabl blocks the activation of DMSO induced apoptosis, demonstrating that BcrAb1 is distinct from v-Ab1 in its ability to block DMSO-induced apoptosis.

Expression of Bcl-2 and Bax in 32Dc13, 32Dc13/v-Ab1, and 32Dc13/BcrAb1 cell lines during induction of apoptosis by DMSO. To further dissect the different effects of ectopic expression of v-ab1 versus bcrabl on DMSO-induced apoptosis of 32Dc13 cells, the expression of Bcl-2 and Bax, gene products known as central players in apoptosis, was analyzed. Towards this end, cell lysates were prepared from the three different cell lines following their culturing in medium containing both IL-3 and 1.5% DMSO, separated by SDS-PAGE and immunoblotted with antiserum Bcl-2 and Bax antibodies. As shown in Fig 2A, Bcl-2 protein expression in 32Dc13 cells began to decrease 24 hours after exposure to DMSO and continued to decline over the duration of the exposure, ultimately to a level barely detectable after 72 hours. In contrast, 32Dc13/v-Ab1 cells displayed a reduced basal level of Bcl-2 expression, compared with the parental cells, that remained constant throughout the exposure period. Finally, it was observed that in 32Dc13/BcrAb1 cells, Bcl-2 basal protein expression was elevated compared with both the parental 32Dc13 and 32Dc13/v-Ab1 cells. These observations together suggested that the endogenous level of Bcl-2 expression may be an indicator of apoptosis induction by DMSO in the different 32Dc13 cells.

To analyze for Bax protein expression, Western blots were again prepared by SDS-PAGE fractionation of lysates isolated from DMSO treated cell lines and immunoprobred with an anti-Bax antiserum. As shown in Fig 2B, parental 32Dc13 cells expressed low levels of Bax that was rapidly upregulated, beginning at 6 hours following DMSO exposure, continuing to increase thereafter. Basal Bax expression in 32Dc13/v-Ab1 cells was elevated compared with normal 32Dc13 cells. Expression followed a similar upregulation also beginning at 6 hours, with relative levels of Bax protein remaining higher than seen in the parental 32Dc13 cells. Finally, steady state expression of Bax in 32Dc13/BcrAb1 cells remained constant and was found to be the highest among the three cell lines, both before and after DMSO treatment. When we analyzed the ratio of Bcl-2 to Bax protein expression, we found a correlation between this ratio and the sensitivity of the cell lines to apoptosis. For example, the ratio seen in 32Dc13 cells becomes less than 1 beginning around 48 hours and continues to decline, whereas the lowering of the ratio to about 1 occurs in the 32Dc13/v-Ab1 over the same time course, while a ratio greater than 1 is maintained in 32Dc13/BcrAb1 cells. Thus, this data suggest that a critical determinant in DMSO-induced apoptosis may be the ratio of Bcl-2 to Bax, as has been previously described.

Apoptosis induced by DMSO in 32Dc13 and 32Dc13/v-Ab1 cells is delayed, but not blocked by Bcl-2 overexpression. Overexpression of Bcl-2 is known to suppress certain apoptotic pathways, but not others. Because 32Dc13/BcrAb1 express elevated levels of Bcl-2 and fail to undergo DMSO-induced apoptosis, we tested the hypothesis that elevated levels of Bcl-2 expression would result in the protection of parental 32Dc13 and 32Dc13/v-Ab1 cells from DMSO-induced apoptosis. To test this possibility we assayed the effect of deregulated overexpression of Bcl-2 protein in both the 32Dc13/v-Ab1 and the parental cell lines. By constitutively overexpressing Bcl-2, we could manipulate the Bcl-2/Bax ratio to favor Bcl-2 to dissect the role of these two gene products.
in DMSO-induced apoptosis. Either an hu–bcl-2 containing expression construct or the expression vector alone was transfected into 32Dc13 and 32Dc13/v-Abl cells by electroporation. Single cell clones were isolated following soft agar plating in medium containing G418 and were shown to express the transfected human Bcl-2 protein by immunoblotting with an antihuman Bcl-2 antibody (Fig 3A). The biological activity of the human Bcl-2 protein in the parental 32Dc13 cells was confirmed by prolonging the survival of 32Dc13/bcl-2 cells in medium devoid of IL-3 (data not shown), as has been previously described.27 32Dc13/neo, 32Dc13/bcl-2, 32Dc13/v-Abl/bcl-2, and 32Dc13/v-Abl/neo cells were then exposed to DMSO to determine their apoptotic response. As shown in Fig 3B, although overexpression of bcl-2 in 32Dc13 cells cultured in the presence of IL-3 and DMSO afforded some protection, resulting in increased survival compared with 32Dc13/neo cells cultured under identical conditions (approximately a 50% increase in viable cells at day 6), it fails to prevent the induction of apoptosis. This inability of Bcl-2 to suppress DMSO-induced apoptosis is similar to the effect of overexpression of Bcl-2 on factor-dependent cells when cultured in the absence of cytokine.27 Because IL-3 was found to increase the survival of 32Dc13/v-Abl expressing cells (Fig 1C), we eliminated IL-3 from the medium to determine the effect of Bcl-2 overexpression alone on cell survival. Figure 3B shows that Bcl-2 provided limited protection, approximately 30% increase in viable cell numbers at day 6. Thus, altering the ratio of Bcl-2 to Bax by increasing Bcl-2 expression does not result in a response similar to that observed when 32Dc13/BcrAbl cells are treated with DMSO. Because Bcl-2 overexpression in 32Dc13/v-Abl cells could not mimic the survival observed in 32Dc13/BcrAbl cells, other gene products besides Bcl-2 must be involved.

We were also interested in comparing the protective effect of Bcl-2 versus IL-3 in 32Dc13/v-Abl cells. We found that IL-3 afforded the greater protection from DMSO-induced apoptosis in these cells. As seen in Fig 3B, survival of 32Dc13/v-Abl cells in the presence of DMSO was enhanced approximately 30% at day 6 by the overexpression of Bcl-2, whereas adding IL-3 to the medium resulted in an almost 50% increase in survival after 6 days exposure to DMSO.

DISCUSSION

To gain insights into the biological properties of the activated oncoproteins v-Abl and BcrAbl, we compared their effect on apoptosis induction using transgenic murine myeloid 32Dc13 cells. We report that although both of these genes encode for an activated tyrosine kinase function, they differ in their ability to suppress apoptosis. We found that in the presence of DMSO, the parental 32Dc13 cells displayed a decrease in cell viability due to the induction of apoptosis. When the parental and transgenic 32Dc13 cell lines were exposed to DMSO, only those expressing bcrabl were blocked from the induction of apoptosis. Analysis of Bel-
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Fig 3. Effect of bcl-2 overexpression on apoptosis in 32Dc13 and 32Dc13/P160 v-Abl cell lines. (A) Stable expression of hu-Bcl-2 in 32Dc13 and 32Dc13/P160 v-Abl transfected with pZip/hu-bcl-2. Cell lysates were prepared from 32Dc13 cells stably transfected with pZip-neo or pZip-hu-bcl-2, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-hu-Bcl-2 antibody. Lane 1, 32Dc13/neo; lane 2, 32Dc13/P160 v-Abl/neo; lane 3, 32Dc13/Bcl-2 #1; lane 4, 32Dc13/Bcl-2 #2; lane 5, 32Dc13/P160 v-Abl/Bcl-2 #1; and lane 6, 32Dc13/P160 v-abl/Bcl-2 #2. (B) Effect of exogenous Bcl-2 expression on DMSO-induced apoptosis in 32Dc13/neo and 32Dc13/P160 v-Abl cell lines. Cell viability was determined by trypan blue exclusion using a hemocytometer. The results are graphed as the means of a minimum of duplicate experiments.

2 and Bax expression showed differences during DMSO-induced apoptosis among the three cell lines that correlated with their response to DMSO. However, overexpression of the bcl-2 transgene slows down, but does not block the induction of apoptosis in either the parental or v-abl expressing cell lines, indicating that, the DMSO-induced apoptotic pathway includes other survival players besides Bcl-2.

The induction of apoptosis in 32Dc13 cells by both DMSO exposure and IL-3 withdrawal in 32Dc13 cells was very similar, albeit the DMSO-induced apoptosis occurred at a slower rate. Exposure of parental 32Dc13 cells to either condition resulted in G1 arrest (data not shown), genomic DNA fragmentation into oligonucleosome ladders, a reversal in the ratio of endogenous expression of Bcl-2 to Bax, and an inability for overexpressed Bcl-2 to block apoptosis27 (Figs 2 and 3). The data we present demonstrate that 32Dc13 cells expressing either v-abl or bcrab in exhibit distinguishing behavior in their response to DMSO-induced apoptosis, in contrast to the shared ability to block apoptosis induction on withdrawal of IL-3. v-Abl oncoprotein is incapable of preventing DMSO-induced apoptotic death, either in the presence or absence of IL-3. Although 32Dc13 cells expressing v-abl demonstrate a limited increase of survival in DMSO compared with the parental cells, they were shown to contain fragmented DNA, indicative of apoptosis. In stark contrast, the cells expressing bcrab are protected from apoptosis induced by DMSO.

We found that either overexpression of Bcl-2 or culturing in the presence IL-3 resulted in a limited enhancement of survival by 32Dc13 parental and v-abl expressing cells on induction of apoptosis by DMSO. The survival promoting properties of IL-3 have been described previously28,29 but within the context of factor-dependent cell lines. Both BcrAbl and v-Abl substitute equally for IL-3 promoting proliferation and survival, however, in the presence of DMSO, only BcrAbl can promote both survival and proliferation. Neither IL-3 or v-Abl can maintain cell survival in the presence of DMSO, although v-Abl appears to provide more protection than IL-3, nor can the combination of v-Abl and IL-3 block apoptosis. Using this system, the kinase activity of BcrAbl is distinguishable from both that of v-Abl and the IL-3 receptor complex. This model has been used to identify different effects of expression of these activated Abl kinases, and will be useful to determine kinase dominance and potentially to identify specific substrates that contribute to the different phenotypes.

Other studies have described the inhibition of apoptosis by BcrAbl30,31 and as this work was in progress, a role for bcl-2 in BcrAbl oncogenicity was proposed, by linking bcl-2 expression to BcrAbl-mediated tumorigenicity.32 Studies with chronic myelogenous leukemia samples have shown the coexpression of bcr/abl and bcl-2, as well.33 We observed an increased expression of Bcl-2 in the 32Dc13/BcrAbl cells, both in single cells clones, as well as the mass transfected cell population (Fig 2 and data not shown) and hypothesized this was responsible for the survival of these cells. This does not appear to be the case for several reasons; 32Dc13/v-Abl cells survive independently of IL-3 without upregulating Bcl-2 expression and the reconstitution experiments with 32Dc13/v-Abl cells and Bcl-2 transgenes. Overexpression of Bcl-2 only results in a slow down in apoptosis, not a block. The upregulation of Bcl-2 may provide some growth advantage, but does not appear to be responsible for the block in apoptosis, unless the sensitivity of BcrAbl cells is unique compared with both the v-Abl and the parental cells. We are currently testing this with antisense Bcl-2 experiments. Other gene products specifically expressed or activated by the BcrAbl kinase signaling pathway could cooperate with Bcl-2 in promoting survival or be responsible for the survival of these cells.

The enhanced kinase activity encoded by these activated Abl proteins is responsible for their oncogenic and biological properties.34 The loss of kinase activity in factor-dependent cells expressing a temperature-sensitive (ts) v-abl construct renders them sensitive to an apoptotic response following factor withdrawal.35 When 32Dc13 cells expressing bcrab are exposed to protein kinase inhibitors, the cells die by a nonapoptotic pathway, whereas the parental cells die by
apoptosis. The differential response we observed between 32Dc13/v-Abl and 32Dc13/BcrAbl cells was not due to a decrease in the kinase activity of p160v-abl during exposure to DMSO (data not shown). Similarly, this difference does not correspond to differences in the level of protein expression of the two gene products. The cDNA inserts were expressed from a common promoter, both proteins were functionally identical in abrogating IL-3 dependence as shown in Fig 2B, and Western blot analysis showed similar expression levels of the proteins in lysates from the genetically engineered 32Dc13 cells (data not shown). These results do show that the inhibition of apoptosis does not result simply from the deregulated intracellular expression of a tyrosine kinase, but rather is a specific property of the encoded gene product.

The different responses of the activated abl gene products can be attributed to the novel 5' ends of their genes. Bcr sequences play an important role in BcrAbl-mediated transformation, they are autophosphorylated, and associate with Grb-2 SH2 domain. It has also been reported that a member of the 14-3-3 protein family associates with BcrAbl through the bcr encoded sequences. Although both activated Abl proteins are excluded from the nucleus, differences exist in their subcellular localization. The v-abl encoded protein is myristylated and localized to the inner face of the cell membrane, while BcrAbl associates with actin filaments. The loss of myristylation by v-abl gag mutants expressed in 32Dc13 cells does not seem to influence their sensitivity to DMSO (data not shown), indicating that membrane localization may not be responsible for the observed difference between 32Dc13/v-Abl and 32Dc13/BcrAbl cells. The phosphorylation of unique substrates due to protein associations through bcr versus gag encoded sequences may play a determining role in the mechanism of the apoptosis inhibition.

In conclusion, we have established a model system and demonstrated that different forms of the activated abl protooncogene exhibit diverse effects on DMSO-induced apoptosis of 32Dc13 cells. The differential effects were shown to correlate independently of the level of Bcl-2 expression, even though Bcl-2 is upregulated in 32D/BcrAbl cells. In addition, we identified a survival property of IL-3 in v-Abl-transformed 32Dc13 cells, which have become independent of IL-3 for normal cell proliferation, but protected the cells from other apoptotic stimuli. The activated abl genes are unfortunately not good models for understanding the role of the normal c-abl gene product because of their constitutively activated tyrosine kinase resulting from their structural modifications. Recent evidence suggests that contrary to inducing proliferation, c-Abl expression results in growth suppression in fibroblasts by inducing G1 arrest. Thus, it will be interesting to also determine the effect of expression of this gene product on apoptotic responses using this 32Dc13 model system.

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Activated abl oncogenes and apoptosis: differing responses of transformed myeloid progenitor cell lines

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