Dominant Negative MYC Blocks Transformation by ABL Oncogenes

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Summary

A link between ABL oncogenes and MYC is suggested by the transformation synergy that is observed when MYC is expressed at high levels. Dominant negative MYC proteins were overexpressed in fibroblasts to determine if MYC complements ABL oncogene transformation or is essential for this process. Transformation by both v-abl and BCR-ABL oncogenes was reduced 5- to 10-fold, whereas transformation by the serine/threonine kinase oncogene v-mos was unaffected. Using a retrovirus construct modified to express BCR-ABL and MYC genes simultaneously, we show that dominant negative MYC suppressed transformation of primary mouse bone marrow pre-B cells by BCR-ABL. These observations demonstrate that c-MYC is essential for transformation and help define the pathway by which these proteins cause transformation.

Introduction

Transformation by receptor and non-receptor tyrosine kinases results from inappropriate or uncontrolled tyrosine phosphorylation of the kinase molecule itself as well as substrates that signal cell growth (Canley et al., 1991; Koch et al., 1991). The precise details of such cytoplasmic signaling pathways are unclear, but the downstream involvement of nuclear proteins that affect transcriptional control and progression through the cell cycle appears likely. Candidate genes encoding nuclear proteins that may play a role in tyrosine kinase–mediated transformation include MYC, FOS, and JUN, whose transcription is activated by some tyrosine kinase oncogenes as well as by growth factor stimulation (Baichwal et al., 1991; Cleve-land et al., 1989; Dean et al., 1987; Fugii et al., 1989).

It is not known if the transcriptional induction of these genes by tyrosine kinase oncogenes plays a critical role in transformation. A clue that MYC may be critical is suggested by the observation that the receptor tyrosine kinase c-fms fails to induce c-MYC expression and is unable to confer growth stimulation by colony-stimulating factor 1 if the major site of autophosphorylation is mutated. Overexpression of additional c-MYC rescues the mutant allele (Roussel et al., 1991). Data such as these suggest that the nuclear proteins whose transcription is induced by tyrosine kinases all contribute separately, but perhaps collectively, to stimulate cell growth (Hunter, 1991). If this is the case, then one or more of these proteins should be essential for proper transmission of the transformation signal. We have explored this question by examining whether MYC is required for transformation by two distinct but related non-receptor tyrosine kinase oncogenes, v-abl and BCR-ABL. Both are naturally occurring leukemogenic oncoproteins derived from the c-abl protein with activated tyrosine kinase domains (reviewed in Kurzrock et al., 1991; Rosenberg and Witte, 1988). In the case of v-abl, the kinase inhibitory SH3 domain of c-abl is deleted during fusion of gag sequences with the ABL gene, thereby allowing the tyrosine kinase of the fusion protein to be active (Jackson and Baltimore, 1988; Muller et al., 1991). In addition, gag sequences provide an N-terminal myristoylation site, which can relocalize c-abl from the nucleus (Van Etten et al., 1989) to the inner surface of the cell membrane, perhaps allowing the protein access to important substrates. BCR-ABL is a related fusion protein generated by the translocation of ABL sequences on chromosome 9 into BCR sequences on chromosome 22. This fusion product retains the SH3 domain and is not myristoylated, yet is an active tyrosine kinase and potent transforming gene. The observation that BCR first exon sequences can bind the ABL SH2 domain (Pendergast et al., 1991) raises the possibility that this binding could activate the kinase by interfering with the adjacent SH3 domain.

Biologically, both proteins are associated with leukemia. BCR-ABL is specifically expressed in the Philadelphia chromosome positive leukemias as a p210 protein in chronic myelogenous leukemia (CML) and a p185 protein in some cases of acute lymphocytic leukemia (ALL) (reviewed in Kurzrock et al., 1991). v-abl is a naturally occurring retrovirus that causes lymphoid as well as other leukemias in mice (reviewed in Rosenberg and Witte, 1988). Retroviruses expressing either oncogene can transform primary mouse bone marrow, resulting in outgrowths of pre-B lymphocytes when cultured in vitro (Whitlock and Witte, 1982; Rosenberg and Baltimore, 1978; McLauchlin et al., 1987).

We chose to study c-MYC as a potentially critical downstream component for three reasons. First, ABL transgenic mouse studies show strong biologic selection for high level MYC expression. Plasmacytomas develop in these mice after a translocation occurs in the murine c-MYC gene, resulting in overexpression of c-MYC (Rosenbaum et al., 1990). Second, transformation by both v-abl and BCR-ABL is dependent on the level of MYC protein. The latency for plasmacytoma formation in the ABL transgenic mice is shortened after a cross with transgenic MYC mice (Rosenbaum et al., 1990). Third, transforming abl oncogenes have been directly linked to the activation of c-MYC expression. The introduction of v-abl into a murine myeloid hematopoietic cell line specifically increases ex-
pression of c-MYC, but not of FOS or of JUN-B, in a tyrosine kinase–dependent manner (Cleveland et al., 1989). To determine if MYC is essential in transformation by abl oncogenes, we overexpressed wild-type or dominant negative mutants of c-MYC in rodent fibroblasts and hematopoietic cells and assessed the effect of these changes on the ability of v-abl or BCR-ABL to transform these cells. We find that c-MYC is a necessary and limiting component in the transformation pathway of v-abl and BCR-ABL for both fibroblasts and hematopoietic cells.

Results

Strategy for Suppression of c-MYC Function

c-MYC appears to function as a transcription factor by forming a DNA-binding heterodimer with a binding partner such as the recently described MAX (human) or myn (mouse) proteins (Blackwood and Eisenman, 1991; Prendergast et al., 1991). (For convenience we will refer to both forms as MAX in this work.) Complex formation is mediated through an interaction between the helix-loop-helix and leucine zipper domains present in each protein. The resulting MYC–MAX complex has been shown to bind avidly to a specific DNA target sequence in vitro (Blackwell et al., 1990; Halazonetis and Kandil, 1991; Prendergast and Ziff, 1991), but the specific target genes relevant to transformation by c-MYC remain undefined.

Mutational analysis has defined two regions of the c-MYC protein that are essential for transformation (Stone et al., 1987). These include an activation domain in the N-terminus, which can stimulate transcription when fused with GAL4 (Kato et al., 1990), and a C-terminal region that includes a DNA-binding basic region adjacent to helix-loop-helix and leucine zipper motifs. Some of these mutants exhibit dominant negative behavior based on the ability to suppress focus formation of rat embryo fibroblasts after cotransformation with wild-type c-MYC and mutant H-ras. These include a deletion within the activation domain (D106-143) (Dang et al., 1989) and a linker insertion just C-terminal to the DNA-binding basic region (In373) (C. Dang, personal communication). A potential, but as yet untested, mechanism for the dominant negative effect of v-abl on the dominant negative lines, because the characteristic. These results were not due to a selective toxic effect of v-abl on the dominant negative lines, because the viability of the cells before plating in agar was equivalent to the level of v-abl kinase activity was somewhat higher in the parental rat-l line and a set of indicator lines 72 hr after retroviral infection. In this particular experiment the level of v-abl kinase activity was somewhat higher than the endogenous rat c-myc protein (Figure 1B). The dominant negative MYC proteins had no obvious toxic effects on cell growth other than to prolong the doubling time modestly from about 24 to 32 hr.

To compare the effect of the various MYC proteins on transformation by ABL oncogenes, the indicator lines were superinfected with either v-abl or p185BCR-ABL and plated in soft agar. Two precautions were taken to ensure that each cell line was infected at comparable efficiency by both v-abl and p185BCR-ABL and plating in soft agar. Two precautions were taken to ensure that each cell line was infected at comparable efficiency by the ABL retroviruses prior to plating in agar. First, the same vial of virus supernatant was used for all cell lines during each experiment so that virus titers were identical. Second, expression of v-abl or BCR-ABL was monitored by either immunohistochemical staining or immunoprecipitation followed by an autophosphorylation assay. Equivalent transfer of the v-abl or BCR-ABL retrovirus and protein expression was observed for all the cell lines. Figure 2 shows an example of v-abl autokinase activity in the parental rat-1 line and a set of indicator lines 72 hr after retroviral infection. In this particular experiment the level of v-abl kinase activity was somewhat higher in the indicator lines than in the parental cells.

In three separate experiments transformation by v-abl was suppressed 4- to 6-fold in cell lines expressing either the dominant negative mutant D106-143 or In373 (Figure 3, top panel). Suppression of transformation was consistently observed for independently derived lines expressing the dominant negative proteins and was a stable characteristic. These results were not due to a selective toxic effect of v-abl on the dominant negative lines, because the viability of the cells before plating in agar was equivalent (greater than 95%). As predicted, transformation by v-abl
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Figure 1. Expression of MYC Proteins in Rat-1 Indicator Lines
(A) and (B) are immunoblots using the human-specific anti-MYC antibody 9E10 (A) and the polyclonal MYC antiserum (B). The far left lane is lysate from the parental rat-1 line, and the remaining lanes are lysates from indicator lines infected with MYC retroviruses and selected in G418. For D106-143, lanes A, B, C, and D represent independently derived clones. Equal amounts of protein as determined by Lowry assay were loaded per lane.

Figure 2. Expression of v-abl Protein after Infection of the MYC Indicator Lines
Three days after infection with v-abl retrovirus, $5 \times 10^5$ cells from each plate were analyzed for v-abl autokinase activity by immunoprecipitation analysis using the antibody pex5. The far right lane shows the absence of detectable v-abl kinase activity in the uninfected rat-1 cells. The open arrow indicates the migration of the v-abl-specific band at about 100 kD.

Figure 3. Soft Agar Colony Formation in Indicator Lines
The colony numbers are expressed as a percentage of colony formation relative to the parental rat-1 line for ease of comparison. The data represent a summary from three experiments for v-abl, four experiments for BCR-ABL, and two experiments for v-mos. In the case of v-abl and BCR-ABL, the number of colonies for the MYC and In105 indicator lines was more than 3-fold higher than the rat-1 control. For v-mos, colony size and number were equivalent in the parental and dominant negative myc lines D106-143 and In373. The actual colony numbers in the parental rat-1 line for each oncogene were 71 (range 45–104) for v-abl, 102 (range 72–126) for BCR-ABL, and 505 (range 450–560) for v-mos. No colonies were observed when the indicator lines were plated without retrovirus infection.

Dominant Negative MYC Suppresses Transformation by v-abl after Simultaneous Infection of Rat-1 Cells
One explanation for the suppressed transformation phenotype in the indicator line experiments could be that chronic overexpression of the dominant negative MYC mu-
Three days after infection with p185BcR-ABL retrovirus, 2.5 × 10⁴ cells from each indicator line were plated in soft agar. Photographs were taken at 3 weeks.

Mutants Lacking the Helix-Loop-Helix and Leucine Zipper Dimerization Domains of c-MYC Do Not Suppress Transformation by v-ABL Oncogenes

To determine if suppression of v-ABL oncogene transformation was specific for the MYC mutants D106-143 and ln373, we tested other mutations in c-MYC previously characterized for their ability to transform fibroblasts in conjunction with H-ras (Stone et al., 1987). As discussed previously, an insertion in an N-terminal region of c-MYC (In105), which preserves an intact activation domain and continues to function in the ras + MYC transformation assay, remained stimulatory for transformation by v-ABL and BCR-ABL. Two other mutants, U37-142 and D414-433, which remove the entire helix-loop-helix and leucine zipper domains, respectively, have been previously shown to be nontransforming in the ras + MYC cotransformation assay (Stone et al., 1987). Because these mutant proteins would be incapable of dimerization, we predicted they would have no effect on transformation by v-ABL or BCR-ABL. Indeed, after superinfection of cell lines expressing either of these proteins with v-ABL or BCR-ABL retrovirus, we observed no difference in colony formation over the fibroblast control (see Figure 3). These data show that MYC mutations lacking the dimerization motif do not suppress transformation by ABL oncogenes, suggesting that suppression results from competition between the mutant MYC and the native c-MYC protein for dimerization with a binding partner such as max.

Transformation by the Serine Kinase Oncogene v-mos Is Not Suppressed by Dominant Negative MYC

One possible explanation for the transformation suppression we observed in the D106-143 or the ln373 indicator lines is that expression of the dominant negative MYC proteins nonspecifically suppressed their growth such that they were incapable of transformation by any oncogene. For this reason we tested the ability of an unrelated oncogene, v-mos, to transform the indicator lines in comparison with the parental rat-1 line. v-mos is a serine kinase that may transform cells by mechanisms distinct from v-ABL and BCR-ABL. In two separate experiments we found that all of the indicator lines, including the two dominant negative lines, were equally susceptible to transformation by retroviruses expressing the v-mos gene. (Figure 4, bottom panel). This result indicates that the transformation of rat-1 cells by v-mos is independent of dominant negative MYC expression.

v-ABL and BCR-ABL-Transformed Cells from the Dominant Negative MYC Indicator Lines Have Reduced or Absent Expression of the Dominant Negative MYC Protein

As shown in Figure 3, a small, but consistent, number
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The naturally occurring BCR-ABL-related malignancies CML and ALL occur exclusively in hematopoietic cells. To determine if MYC also plays a role in BCR-ABL transformation of hematopoietic cells, we tested the ability of dominant negative MYC proteins to suppress transformation of pre-B cells by BCR-ABL using an in vitro bone marrow transformation assay (McLaughlin et al., 1987, 1989). By using primary bone marrow cells instead of established cell lines, we also could control for any potentially confounding effect that immortalized cell lines might have on the interpretation of the results.

As with the fibroblast work discussed above, our strategy for interfering with MYC function was to overexpress dominant negative MYC mutations. The lower efficiency of retroviral infection of bone marrow cells in comparison with fibroblasts forced us to modify the experimental design to ensure successful delivery of both BCR-ABL and a MYC gene to the same cell. This was achieved using the ACR-ARI--MYC retroviral vector design described above. Wild-type c-MYC or each of the two dominant negative
MYC mutants were paired with p185BCR-ABL in the modified vector and used to generate retrovirus stocks by COS-7 cell transfection. Control plasmids included retrovirus constructs expressing BCR ABL with the G418 resistance gene or BCR-ABL with the In105 MYC mutant. Transformation of mouse bone marrow cells was measured by growing the cells in liquid culture for a period of 3–5 weeks. In this assay cultures infected with p185BCR-ABL retrovirus reproducibly reached a density of 5 × 10^3 to 20 × 10^3 cells per ml within 2–3 weeks as compared with less than 2 × 10^3 to 3 × 10^3 cells per ml for control plates (McLaughlin et al., 1989). In addition, the BCR-ABL-transformed cultures can be distinguished by the larger size of the pre-B cells and the need to feed the cultures more frequently due to acidification of the media. As with the fibroblast assay, overexpression of c-MYC alone is not transforming in this assay (Schwartz et al., 1986).

If overexpression of the dominant negative MYC protein suppressed transformation of bone marrow cells by BCR-ABL, we would expect that cultures infected with BCR-ABL-MYC viruses that contained the D106-143 or In373 MYC mutants would not reach a high cell density. Cultures infected with BCR-ABL-MYC viruses containing wild-type MYC or the In105 mutant should be transformed within 3 weeks. To be sure that the titers of the BCR-ABL-MYC virus stocks were equal, they were compared for their ability to transfer the BCR-ABL gene to fibroblasts. Equivalent numbers of cells stained positive for expression of BCR-ABL for each virus stock in NIH 3T3 cells 3 days after infection (data not shown).

In four experiments using the BCR-ABL-MYC viruses (Table 2), 37 of 40 bone marrow cultures that were infected with retrovirus expressing BCR-ABL-neo or BCR-ABL with wild-type MYC or the In105 mutant reached a density greater than 5 × 10^9 cells/ml within 3 weeks. In contrast, only 1 of 24 plates infected with the BCR-ABL-MYC viruses that contained the dominant negative MYC genes was transformed within 3 weeks. Most of these cultures never developed a pre-B cell outgrowth even after 6 weeks in culture, indicating complete suppression of BCR-ABL transformation (Figure 8). However, 5 of 24 cultures did develop BCR-ABL outgrowths after 6 weeks in culture. In comparison with the BCR-ABL-neo or BCR-ABL-c-MYC-infected dishes, this longer latency most likely reflects the growth of rare clones that have escaped transformation suppression.

Discussion

We have demonstrated that overexpression of dominant negative mutations of c-MYC suppresses transformation by BCR-ABL and v-abl. Suppression of transformation was a consistent phenotype both for dominant negative mutants and in multiple independently derived fibroblast lines expressing the mutants. By performing experiments in which both a dominant negative MYC and a v-abl gene were simultaneously introduced into the target cells, we have also shown that the suppression of transformation phenotype was not a result of unrelated mutations in the established indicator lines. Finally, dominant negative MYC proteins suppressed transformation by v-abl oncogenes but not by the serine kinase v-mos oncogene, indicating specificity in transformation suppression. Our
findings show that MYC is essential for transformation of fibroblasts and hematopoietic cells by v-abl and BCR-ABL.

The discovery of the MYC-binding partner MAX (Blackwood and Eisenman, 1991; Prendergast et al., 1991) suggests a mechanism by which these two mutant MYC proteins exert their dominant negative effect. The dominant negative MYC proteins have mutations located in the domains required for transcriptional activation (D106-143) or DNA binding (In373), but both have intact dimerization motifs. Because MYC and MAX bind via the helix-loophelix and leucine zipper domains of each protein, the mutant proteins could compete with endogenous c-myc for binding to MAX and form nonfunctional heterodimers. This model is supported by the observation that overexpressed MYC proteins with deletions of the dimerization domain (D371-412 and D414-433) did not suppress transformation and that introduction of additional MYC rescues the suppressed transformation phenotype. Importantly, because MAX can also bind to n-MYC (Blackwood and Eisenman, 1991), the mutant proteins could also compete with n-MYC for binding to MAX.

There is precedent from studies of other oncogenes that MYC plays a critical role in transformation pathways. Overexpression of anti-sense MYC in fibroblasts transformed by RAS causes reversion of the transformed phenotype, suggesting that MYC is a necessary downstream effector of RAS transformation (Sklar et al., 1991). A colony-stimulating factor 1 receptor mutant that lacks the major autophosphorylation site can transmit a colony-stimulating factor 1-mediated growth stimulus only if c-MYC is overexpressed (Roussel et al., 1991). Other nuclear proto-oncogenes also play essential roles in transformation by cytoplasmic oncogenes. A dominant negative JUN protein blocks transformation of NIH 3T3 fibroblasts by RAS (Lloyd et al., 1991). Related studies implicate RAS as a necessary component in tyrosine kinase transformation pathways. Focus formation of fibroblasts by SRC, a cytoplasmic tyrosine kinase closely related to ABL, is blocked by microinjection of either antibodies to RAS (Smith et al., 1986) or dominant negative forms of RAS (Stacey et al., 1991), or by overexpression of the GTPase-activating protein (GAP) (DeClue et al., 1991; Nori et al., 1991).

The demonstration that MYC is essential for transformation by ABL oncogenes forces us to consider how these two genes might be connected. The conclusions about signaling pathways that can be made using dominant negative studies are limited without precise knowledge of a physical or biochemical relationship between the two components being studied. Nonetheless, together with the observations linking SRC and RAS, RAS and MYC, and c-fms and MYC, our findings are consistent with a model in which MYC is downstream in the transformation signal of BCR-
ABL and v-abl. This model would state that the increase in MYC expression induced by v-abl (Cleveland et al., 1989) and BCR-ABL (C. L. S. and O. N. W., unpublished data) is a critical downstream event in ABL oncogene transformation and that dominant negative MYC suppresses transformation by blocking the effect of the increased MYC protein. The observation that transformation by ABL oncogenes is increased by additional MYC can be explained if MYC is also a limiting component in the transformation pathway.

An alternative model that explains the transformation synergy observed for these two oncogenes is one in which MYC and ABL oncogenes signal through independent pathways. An important distinction between these two genes in the transformation assays used in this study is that BCR-ABL and v-abl are fully competent for transformation whereas MYC is not. If BCR-ABL and MYC are on independent pathways, the fact that disruption of MYC function blocks ABL oncogene transformation states that the MYC pathway is required for the ABL oncogene pathway.

A specific effect of ABL oncogene expression on MYC will be required to distinguish between these models. Enhanced transcription from the MYC locus mediated by ABL oncogenes is one such effect. However, an increase in MYC protein cannot be the sole transforming effect of ABL oncogenes because MYC overexpression does not substitute for the ABL oncogene transformation phenotype. One possibility is that ABL oncogenes qualitatively alter the MYC protein to modify its biological activity. Other components in addition to MYC also may be required to reconstitute the ABL oncogene transformation signal because ABL oncogenes may activate multiple pathways that are required for transformation. The identification of ABL oncogene mutants that incorporate parts of the transformation pathway may allow the identification of these additional components by complementation studies.

The enhanced in vitro transforming ability of BCR-ABL in the setting of increased MYC suggests a potential role for MYC in the progression of GML from the chronic phase to the more malignant blast crisis. Translocation, amplification, and overexpression of the c-MYC gene have been reported in some patients with blast crisis of CML and overexpression of the c-MYC gene have been associated with proliferation of COS-7 cells (McLaughlin et al., 1987). The MYC cDNAs were subcloned into the EcoRI site of pBluescript (Muller et al., 1991) to allow preparation of virus stocks by transient transfection of COS-7 cells. The virus stocks were characterized by infection of NIH 3T3 cells followed by immunoprecipitation of the ABL protein and autokinase assay. MYC virus stocks were characterized by transfer of G418 resistance to NIH 3T3 cells. Titers were in the range of 10⁶ virus particles per ml such that 20%–40% of the target fibroblasts were infected in a typical experiment.

**Generation of Indicator Lines and Soft Agar Colony Assays**

The MYC indicator lines were generated by infection of rat-1 cells with helper-free retrovirus followed by selection in Q410 (0.5 mg/ml) for 3–4 weeks. Individual G418-resistant colonies as well as a pooled population of G418-resistant cells were screened for MYC protein expression and expanded. All indicator lines were maintained continuously in G418 to prevent the growth of cells that might have deleted the retroviral construct.

**Transformation by v-abl and BCR-ABL** was measured using a soft agar assay as described (Lugo and Witte, 1989). The indicator lines were plated at a density of 4 x 10⁵ cells/10 cm² dish overnight. Infection was performed for 5 hr at 37°C using 1 ml of virus stock with 8 μg/ml polybrene. Forty-eight hours later, 5 x 10⁴ cells from each dish were analyzed by immunohistochemical staining with the anti-ABL monoclonal antibody Q110 (Muller et al., 1991) to confirm equal transfer of the retroviral construct.

**Murine Bone Marrow Pre-B Cell Assay**

Fresh bone marrow from the femurs and tibias of 4-week-old BALB/c mice was infected with retrovirus and plated at a density of 5 x 10⁵ cells/6 cm² dish in duplicate, dishes were refed at 1 week, and colonies counted at 2.3 weeks. Colonies greater than 0.75 mm in size were scored positive.

**Protein Analysis**

Expression of MYC proteins was measured by Western analysis using the monoclonal antibody 9E10 (Oncogene Science), which recognizes the MYC C-terminus and is specific for human c-MYC (Evan et al., 1985). In some cases, a rabbit polyclonal antibody to bacterially synthesized c-MYC (a gift of Chi Dang, Johns Hopkins School of Medicine) was used to recognize MYC mutants not recognized by 9E10. Cell pellets were lysed in 10 mM Tris (pH 7.4) and 1% SDS at 100°C and analyzed as described (Muller et al., 1991). Autokinase assays for BCR-ABL and v-abl were performed as previously described (Koropka and Witte, 1989).
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