Current Biology, Vol. 13, 437–441, March 4, 2003, ©2003 Elsevier Science Ltd. All rights reserved. PII S0960-9822(03)00090-3

The c-Myc Oncoprotein Interacts with Bcr

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Summary

Bcr is a multifunctional protein that is the fusion partner for Abl (p210 Bcr-Abl) in Philadelphia chromosome positive leukemias. We have identified c-Myc as a binding partner for Bcr in both yeast and mammalian cells. We are also able to observe interactions between natively expressed c-Myc and Bcr in leukemic cell lines. Although Bcr and Max have overlapping binding sites on c-Myc, Bcr cannot interact with Max, or with the c-Myc•Max heterodimer. Bcr expression blocks activation of c-Myc-responsive genes, as well as the transformed phenotype induced by coexpression of c-Myc and H-Ras, and this finding suggests that one function of Bcr is to limit the activity of c-Myc. However, Bcr does not block c-Myc function by preventing its nuclear localization. Interestingly, increased Bcr dosage in COS-7 and K-562 cells correlates with a reduction in c-Myc protein levels, suggesting that Bcr may in fact be limiting c-Myc activity by regulating its stability. These data indicate that Bcr is a novel regulator of c-Myc function whose disrupted expression may contribute to the high level of c-Myc protein that is observed in Bcr-Abl transformed cells.

Results and Discussion

p160 Bcr Interacts with c-Myc in Yeast

In order to identify new binding partners for p160 Bcr, a yeast two-hybrid screen was performed. Multiple independent copies of four structurally unrelated clones were identified that interacted with a full-length p160 Bcr bait. One of these clones encodes a carboxy-terminal fragment of c-Myc (residues 103–439) that includes the complete Myc box II sequences as well as an intact basic/helix-loop-helix/leucine zipper (B/HLH/Z) domain. Our efforts to more precisely define the binding site for c-Myc with an extended panel of Bcr baits revealed an interaction between c-Myc and a Bcr fragment containing residues 871–910 that encompasses the small region between the PH domain and the C2 domain (Figure 1A).

In order to identify the region of c-Myc that interacts with Bcr, a panel of derivatives was constructed that contained different fragments of the c-Myc protein (Figure 1B), and each was tested for its ability to interact with full-length Bcr. The smallest fragment of c-Myc that we have identified that retains its ability to interact with the full-length Bcr clone consists of the carboxy-terminal B/HLH/Z domain.

Full-Length c-Myc Interacts with p160 Bcr in Mammalian Cell Lines

Next, we wished to determine whether Bcr interacts with full-length c-Myc, and whether this interaction can be recapitulated in mammalian cells. To address these questions, we coexpressed full-length c-Myc and hemagglutinin-tagged (HA) full-length Bcr in COS-7 cells and performed coimmunoprecipitations (Figure 2A) with either anti-HA (F7, Santa Cruz) or anti-Myc (C-33, Santa Cruz) monoclonal antibodies. In either case, we were able to detect an interaction between full-length Bcr and c-Myc. To confirm that c-Myc interacts with the carboxyl terminus of Bcr, we then repeated the coimmunoprecipitation with two smaller nonoverlapping fragments of Bcr (Figure 2B). Consistent with what we observed in yeast, we were able to precipitate c-Myc with a Bcr fragment that encoded the carboxyl terminus of Bcr (residues 871–1271), but not a fragment that encodes the amino terminus (residues 1–871). To further confirm that the interaction between Bcr and c-Myc is direct, we purified Baculovirus-expressed, full-length FLAG-tagged c-Myc from insect Sf9 cells and performed an in vitro affinity precipitation with bacterially expressed GST-Bcr(871–1271). A Western blot with an anti-Myc monoclonal antibody (C-33, Santa Cruz) demonstrated an interaction between c-Myc and GST-Bcr(871–1271), but not between c-Myc and GST alone (Figure 2C).

Since the putative Bcr binding site for c-Myc is present within p210 Bcr-Abl, c-Myc and p210 Bcr-Abl may also interact. However, in cotransfection experiments, we were unable to coimmunoprecipitate p210 Bcr-Abl with an anti-Myc monoclonal antibody (C-33, Santa Cruz), despite the fact that we were able to coimmunprecipitate p160 Bcr under the same experimental conditions (Figure 2D).

To determine whether the interaction between full-length Bcr and c-Myc occurs in a more physiologically relevant context, the association between natively expressed proteins was also examined in K-562 and HL-60 cells (Figure 2E). K-562 is a human myeloid cell line that was isolated from a patient with chronic myelogenous leukemia (CML) and is positive for p210 Bcr-Abl. HL-60 cells are a leukemic cell line of myeloid origin that does not contain Bcr-Abl but expresses high levels of c-Myc. In both instances, we were able to coimmunoprecipitate p160 Bcr with a monoclonal antibody specific for c-Myc (C-33; Santa Cruz; Figure 2D, top panel).

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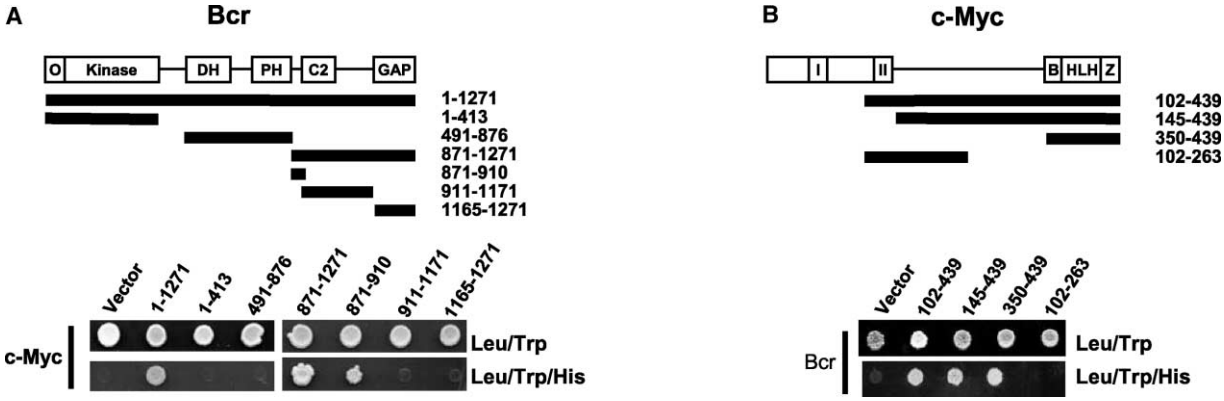

Figure 1. Yeast Two-Hybrid Assays Identify c-Myc as a Binding Partner for p160 Bcr

(A) Mapping of the c-Myc binding site within p160 Bcr. The domain structure of the full-length Bcr protein is illustrated in the upper line (O, oligomerization domain; kinase, serine/threonine kinase domain; DH, Dbl homology domain; PH, pleckstrin homology domain; C2, calcium binding domain; GAP, GTPase activating protein domain), and the lines below indicate the regions of the protein included in predicted translation products of the various cDNA derivatives. In the lower panels, yeast colonies that grew on plates lacking leucine and tryptophan (Leu/Trp) were examined for growth on histidine-deficient plates (Leu/Trp/His). Growth in the absence of leucine indicates the presence of pGAD containing residues 102–439 of c-Myc, while growth in the absence of tryptophan indicates the presence of pGBT9 containing the indicated derivatives of Bcr. Interactions between proteins are demonstrated by the ability to activate the *HIS3* reporter gene.

(B) Full-length Bcr binds to the carboxy-terminal B/HLH/Z domain of c-Myc. The domain structure of the full-length c-Myc protein is illustrated in the upper line (I and II, Myc boxes I and II; b, basic domain; HLH, helix-loop-helix fold; Z, leucine zipper), and the lines below indicate the regions of the protein included in predicted translation products of the various cDNA derivatives. In the lower panels, interactions between full-length p160 Bcr and the indicated derivatives of c-Myc were determined as described above.

The specificity of this interaction was confirmed by our failure to precipitate Bcr with an irrelevant HA antibody (F7; Santa Cruz; second panel from top). The c-Myc antibody did not coimmunoprecipitate p210 Bcr-Abl from K-562 cells, which is consistent with what we observed in our overexpression experiments (see Figure 2D).

Bcr Limits c-Myc Transactivation and Transforming Activity

Since c-Myc has been best characterized as a transcriptional regulator, we wondered whether the interaction with Bcr would influence c-Myc-mediated transcriptional activity. Using in vitro oligonucleotide binding assays, it has been shown that a c-Myc•Max hetero-

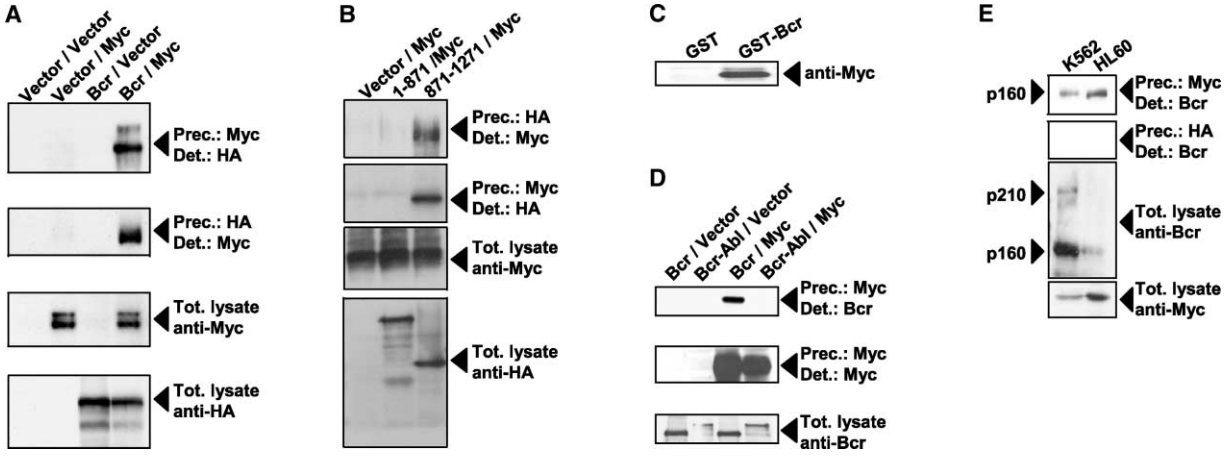

Figure 2. Full-Length c-Myc Interacts with p160 Bcr in Mammalian Cells

(A–E) Prec. indicates the antibody used in immunoprecipitations, and Det. indicates the antibody used in a Western blot to detect an interaction. (A, B, and D) COS-7 cells were cotransfected with the indicated combinations of plasmids (vector, pAX142; Bcr, pAX142-*bcr*(1–1271); Myc, pAX142-*myc*; 1–871, pAX142-*bcr*(1–871); 871–1271, pAX142-*bcr*(871–1271); Bcr-Abl, pAX142-*bcr-abl*). Lysates were collected at 48 hr and were examined by Western blot for expression of the appropriate plasmids (Tot. lysate). Immunoprecipitations were then performed with the indicated antibodies to detect an interaction. (A) Full-length c-Myc interacts with p160 Bcr. (B) c-Myc interacts with the carboxyl terminus of Bcr. (C) Full-length FLAG-myc protein interacts with GST-Bcr(871–1271) in an in vitro affinity precipitation assay. Baculovirus-expressed FLAG-myc protein was purified from Sf9 cells, and then incubated with bacterially purified sepharose-linked GST or GST-Bcr(871–1271). The beads were then washed, and an interaction was demonstrated by Western blot with an anti-Myc antibody. (D) Full-length c-Myc does not interact with p210 Bcr-Abl. (E) Natively expressed Bcr interacts with natively expressed c-Myc in hematopoietic cell lines. Lysates were collected from K-562 and HL-60 cells and were examined for expression of c-Myc and p160 Bcr (lower two panels). Lysates were then precipitated with either anti-Myc (upper panel) or an irrelevant anti-HA (second panel) antibody, and interactions were demonstrated by Western blot with an anti-Bcr antibody that recognizes the amino terminus of both p160 Bcr and p210 Bcr-Abl (upper panel).

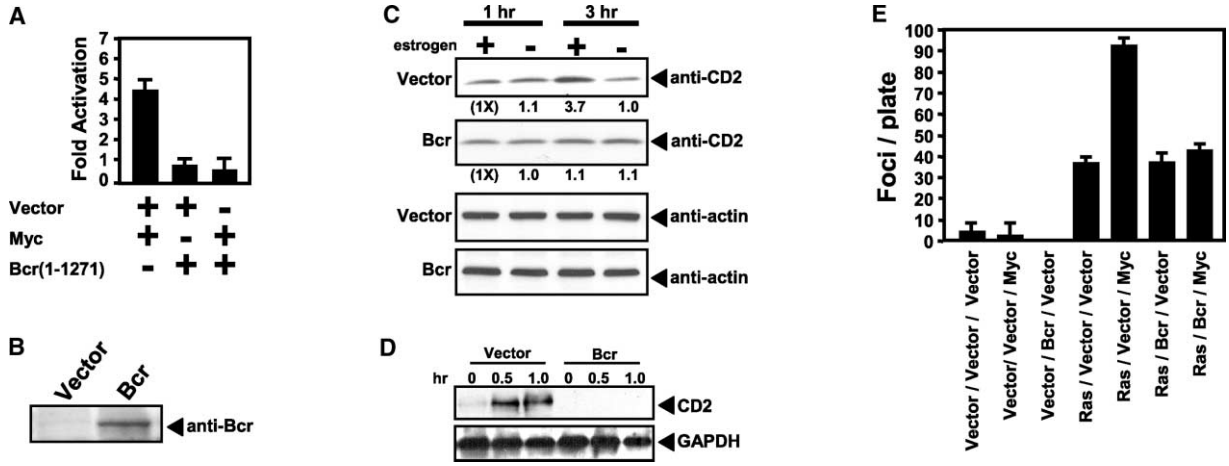

Figure 3. Bcr Limits c-Myc Transactivation and Transforming Activity

(A) p160 Bcr blocks activation of c-Myc response elements by c-Myc. COS-7 cells were cotransfected (3 μg of each plasmid) with the indicated combinations of pAX142 (vector), pAX142-*bcr*(1–1271), or pAX142-*myc*, along with 2.5 μg pMyc-TA-*luc* (Clontech),and 500 ng pCMVnlac as an internal control for transfection efficiency and/or growth inhibition. Luciferase assays were performed as described in the Experimental Procedures. Data shown are representative of three independent experiments performed on triplicate plates. Error bars indicate standard deviations.

(B) Western blot showing stable expression of p160 Bcr in mycER (Rat1a) cells.

(C and D) p160 Bcr blocks transcriptional activation of cyclin D2 (CD2) by c-Myc. mycER cells that express either vector or Bcr were serum starved (0.25% charcoal stripped FCS) for 24 hr, and then c-Myc was induced with 2 μM β-estradiol (estrogen). (C) Lysates were collected at 1 hr and 3 hr and were examined by Western blot for expression of CD2 and β-actin. (D) Total mRNA was collected at 0, 0.5, and 1.0 hr and was examined by Northern blot for expression of CD2 and GAPDH.

(E) Bcr blocks c-Myc focus formation in NIH 3T3 cells. NIH 3T3 cells were cotransfected with combinations of pAX142 (vector) or pAX142 that encodes the indicated proteins (Myc, pAX142-*myc* [3 μg]; Bcr, pAX142-*bcr*(1–1271) [3 μg]; Ras, pAX142-*ras*(12V) [100 ng]). Primary focus formation assays were performed as described in the Experimental Procedures, and foci were counted at 14 days. Data shown are the average of three assays performed on duplicate plates.

dimer is capable of recognizing the core sequence CA(C/T)GTG, termed the E-box [1–3]. c-Myc is capable of transactivating artificial constructs that contain tandem E-box sequences in the promoters of reporter genes [4]. In our analysis, we utilized a c-Myc-responsive reporter plasmid that contains the gene for luciferase fused at the amino terminus to E-box elements (pMyc-TA-*luc* [Clontech]). When we expressed c-Myc alone with this reporter, we observed a 4-fold increase in luciferase activity (Figure 3A), which is consistent with what has been observed previously [4]. Activation of the reporter was blocked by coexpression with full-length Bcr, suggesting that Bcr can limit c-Myc transcriptional activity.

To further examine the consequences of Bcr expression on c-Myc transcriptional activity, we examined the expression of an endogenous c-Myc target gene in the presence of Bcr. For this analysis, we established mycER (Rat1a) cell lines that were stably transfected with either vector or full-length Bcr (Figure 3B). It has been shown previously that cyclin D2 expression is elevated in response to c-Myc induction in this cell type [5], and, consistent with this, we observed elevated levels of cyclin D2 protein (Figure 3C, 3.7-fold) and mRNA (Figure 3D) in our vector-expressing cells when c-Myc was induced with estrogen. Stable expression of full-length Bcr blocks cyclin D2 induction, again suggesting that Bcr can block transactivation of target genes by c-Myc.

To determine whether Bcr can influence cellular activities that accompany deregulated c-Myc expression, we also examined the effects of Bcr expression on the transforming activity of c-Myc in NIH 3T3 cells. Although c-Myc is not transforming when expressed alone in NIH 3T3 cells, it will cooperate with a constitutively activated mutant of Ras (Ras(12V)) in a primary focus formation assay (Figure 3E, [6, 7]). Coexpression of full-length Bcr with c-Myc and Ras(12V) completely blocked the contribution of c-Myc to Ras(12V) transformation. When expressed in the absence of c-Myc, Bcr did not inhibit Ras(12V) transformation or transformation by an unrelated oncogene (Dbl, not shown), and this finding again suggests that Bcr acts by specifically inhibiting c-Myc function.

c-Myc Protein Levels Are Sensitive to Bcr Dosage

Next, we wished to investigate the mechanism through which Bcr can limit c-Myc function. Although c-Myc does not form homodimers in vivo, heterodimers consisting of c-Myc and its protein partner Max recognize specific DNA sequences contained within the promoters of multiple cellular genes [8]. Since the binding of Max to c-Myc occurs through their respective B/HLH/Z domains, we wondered whether Bcr inhibits c-Myc by interfering with this interaction. Although we were readily able to detect an interaction between overexpressed c-Myc and either endogenous or overexpressed Max in COS-7 cells (Figure 4A), we were unable to detect an interaction between Bcr and Max (Figure 4B). This suggests that the interaction between Bcr and c-Myc is specific and does not extend to all B/HLH/Z proteins. When we performed a triple transfection with Bcr, Max, and c-Myc (Figure 4C), we were unable to detect an

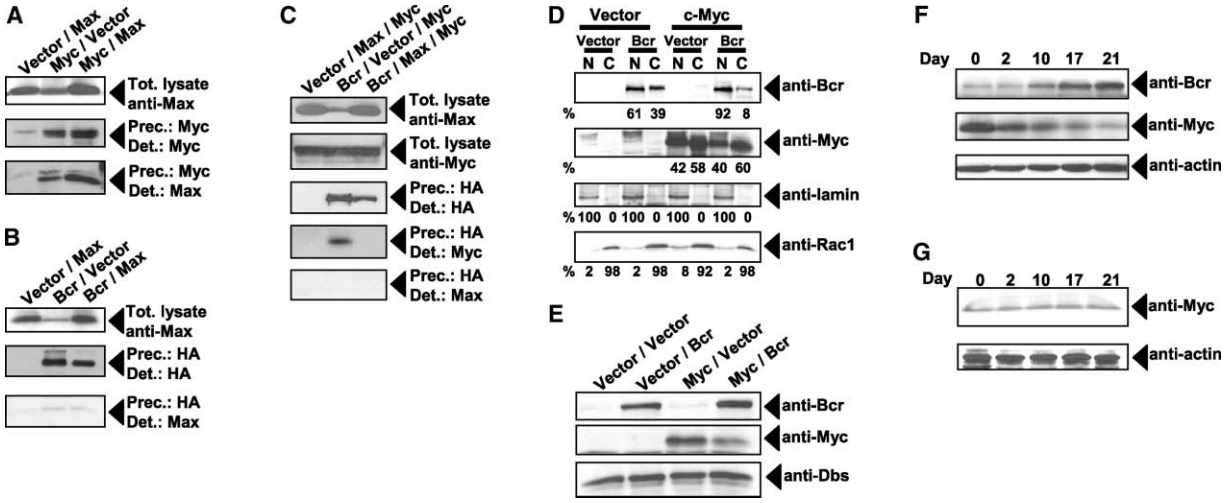

Figure 4. c-Myc Protein Levels Are Sensitive to Bcr Dosage

(A–C) Bcr does not bind Max or the c-Myc•Max heterodimer. COS-7 cells were cotransfected with the indicated combination of plasmids. Vector is empty pAX142, while Myc, Max, and Bcr are pAX142-*myc*, pAX142-*max*, and pAX142-*bcr*(1–1271), respectively. Immunoprecipitations were performed as described in Figure 2.

(D) p160 Bcr does not prevent nuclear accumulation of c-Myc. COS-7 cells were cotransfected with the indicated combinations of plasmids. At 48 hr, lysates were collected and separated into nuclear (N) and cytoplasmic (C) fractions as described in the Experimental Procedures. A total of 25% of the nuclear lysates and 10% of the cytoplasmic lysates were compared for each condition. Protein expression was determined by Western blot with the indicated antibodies. The relative amounts of protein in matched nuclear and cytoplasmic fractions (%) were determined with a PhosphorImager (Molecular Dynamics).

(E) c-Myc protein levels are sensitive to Bcr dosage in COS-7 cells. COS-7 cells were cotransfected with the same combination of plasmids as in (D), except pAX142-*dbs*(HA6) was also included as a control for transfection efficiency. Lysates were collected at 48 hr, and protein expression was determined by Western blot with the indicated antibodies.

(F) Increased p160 Bcr dosage in K-562 cells is associated with decreased levels of c-Myc. Lysates were collected from the K12 K-562 Tet-off clone at different time points after removal of the Bcr tet transcription block. Lysates from the different time points were analyzed by Western blot for expression of p160 Bcr, c-Myc, and β-actin (as an internal control).

(G) Control K-562 cells were maintained on tetracycline for 14 days, and then lysates were collected after Tet removal for the indicated time points. Lysates were examined by Western blot for expression of c-Myc or β-actin (as an internal control). Control K-562 cells were maintained on tetracycline for 14 days, and then lysates were collected after Tet removal for the indicated time points. Lysates were examined by Western blot for expression of c-Myc or β-actin.

interaction between c-Myc and Bcr, even though we are able to detect the interaction in the absence of overexpressed Max (compare lanes 2 and 3). Collectively, these observations suggest that the binding of c-Myc to Max and Bcr may be mutually exclusive events and that Bcr cannot efficiently compete with Max for binding to c-Myc.

To determine whether Bcr may block c-Myc transcriptional activity by preventing its translocation to the nucleus, we examined the nuclear and cytoplasmic fractions of COS-7 cells that coexpress Bcr and c-Myc (Figure 4D). Surprisingly, we were able to detect high levels of Bcr in the nuclear fraction of COS-7 cells even in the absence of c-Myc expression (upper panel). This could not be attributed to cross-contamination of the fractions since the nuclear protein lamin and the cytoplasmic protein Rac1 were efficiently partitioned in the same lysates (lower 2 panels). When coexpressed with c-Myc, we consistently observed a much higher fraction of Bcr in the nucleus (92% versus 61%), suggesting that c-Myc can contribute to the nuclear localization of Bcr. c-Myc was also detected in both the nuclear and cytoplasmic fractions, and the relative concentrations did not change appreciably in response to Bcr expression. However, the overall levels of both nuclear and cytoplasmic c-Myc were consistently lower in the presence of Bcr, and this effect was even more apparent when we examined whole-cell lysates (Figure 4E). This reduction in protein levels could not be attributed to cotransfection efficiency since an internal control for transfection efficiency (Dbs) was expressed equally in all conditions (Figure 4E). Thus, although Bcr does not appear to modify the cellular partitioning of c-Myc, it may have an effect on the overall levels of c-Myc expression.

Since c-Myc protein levels appear to be responsive to Bcr dosage in COS-7 cells, we wondered whether Bcr dosage may also influence the steady-state levels of c-Myc that are present in Bcr-Abl-positive cell lines. To address this possibility, we made use of a K-562 cell line that contains a conditional allele of full-length Bcr. Our previous studies established an inducible *BCR* gene expression system in clones of K-562 cells containing a tetracycline(Tet)-off *BCR* gene [9]. The Bcr Tet-off system is characterized by strong induction of p160 Bcr after removal of tetracycline from the culture medium (Figure 4F, upper panel). Release from the Tet block in the Tet-off clone of K-562 cells induced expression of Bcr that is readily detectable by day 10 and reaches a maximum by day 21. Examination of the same lysates revealed a dramatic reduction in levels of c-Myc that was first detectable by day 2 and continued through day 21 (Figure 4F, middle panel). This reduction was

verified in three independent release experiments conducted on two independent cell clones (data not shown). The reduction could not be attributed to nonspecific reductions in levels of protein expression since β-actin levels remained constant throughout the course of the experiment (Figure 4F, lower panel). In addition, alterations in c-Myc levels could not be attributed to a nonspecific activity associated with tetracycline withdrawal since control K-562 cells that had been maintained on tetracycline for 14 days exhibited no changes in c-Myc levels upon tetracycline withdrawal (Figure 4G, upper panel). These observations suggest that c-Myc levels are sensitive to Bcr dosage in K-562 cells and are consistent with the lower levels of c-Myc expression that we observed in COS-7 cells when Bcr is coexpressed (Figure 4E).

Conclusions

Elevation in the levels of c-Myc is a widespread phenomenon in a large variety of human tumors, and there is a strong feeling that the deregulated expression of c-Myc contributes substantively to the progression of many malignancies [10]. Not surprisingly, c-Myc is also thought to play an important role in the progression of CML. Levels of c-Myc are elevated in Bcr-Abl transformed cells [11], and dominant inhibitory derivatives of c-Myc can effectively block Bcr-Abl transformation [12]. Our observation that c-Myc levels are responsive to Bcr dosage in Bcr-Abl transformed cells suggests a direct mechanism by which c-Myc protein levels may become elevated in Bcr-Abl transformed cells. The contribution of this activity to the transforming potential of Bcr-Abl, particularly within the context of CML progression, is currently under investigation.

Supplementary Material

Supplementary Material including additional methodological details is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

This work was supported by National Institutes of Health grants CA77493 (I.P.W.) and CA49639 (R.B.A.), Cancer Center support grant CA16672 (R.B.A.), and Project 7 (R.B.A.).

Received: May 30, 2002
Revised: January 8, 2003
Accepted: January 8, 2003
Published: March 4, 2003

References

1. Solomon, D.L., Amati, B., and Land, H. (1993). Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers. Nucleic Acids Res. *21*, 5372–5376.
2. Prendergast, G.C., and Ziff, E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science *251*, 186–189.
3. Blackwell, T.K., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science *250*, 1104–1110.
4. Claassen, G.F., and Hann, S.R. (1999). Myc-mediated transformation: the repression connection. Oncogene *18*, 2925–2933.
5. Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J. *18*, 5321–5333.
6. Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature *304*, 596–602.
7. Ruley, H.E. (1990). Transforming collaborations between ras and nuclear oncogenes. Cancer Cells *2*, 258–268.
8. Grandori, C., Cowley, S.M., James, L.P., and Eisenman, R.N. (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu. Rev. Cell Dev. Biol. *16*, 653–699.
9. Lin, F., Monaco, G., Sun, T., Liu, J., Lin, H., Stephens, C., Belmont, J., and Arlinghaus, R.B. (2001). BCR gene expression blocks Bcr-Abl induced pathogenicity in a mouse model. Oncogene *20*, 1873–1881.
10. Nesbit, C.E., Tersak, J.M., and Prochownik, E.V. (1999). MYC oncogenes and human neoplastic disease. Oncogene *18*, 3004–3016.
11. Sawyers, C.L. (1993). The role of myc in transformation by BCR-ABL. Leuk. Lymphoma *11*, 45–46.
12. Sawyers, C.L., Callahan, W., and Witte, O.N. (1992). Dominant negative MYC blocks transformation by ABL oncogenes. Cell *70*, 901–910.