Increased Expression of Bcl-xL and c-Myc Is Associated with Transformation by Abelson Murine Leukemia Virus*

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E. Jacintha Noronha‡, Karen Hinrichs Sterlings§, and Kathryn L. Calame¶¶
From the Departments of §Microbiology and ¶Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, New York 10032

Transformation mediated by the v-Abl oncoprotein, a tyrosine kinase encoded by the Abelson murine leukemia virus (A-MuLV), is a multi-step process requiring genetic alterations in addition to expression of v-Abl. Loss of p53 or p19ARF was previously shown to be required for Abelson murine leukemia virus transformation of primary mouse embryonic fibroblasts (MEFs). By comparing gene expression patterns in primary p53−/− MEFs acutely infected with the v-Abl retrovirus, v-Abl-transformed MEF clones, and v-Abl-transformed MEF clones treated with Abl kinase inhibitor STI 571, we have identified additional genetic alterations associated with v-Abl transformation. Bcl-xL mRNA was elevated in three of five v-Abl-transformed MEF clones. In addition, elevated expression of c-Myc mRNA, caused either by c-myc gene amplification or by enhanced signaling via STAT3, was observed in five v-Abl-transformed MEF clones. The data suggest that increases in cell survival associated with Bcl-xL and increases in cell growth associated with c-Myc facilitate the transformation process dependent on constitutive mitogenic signaling by v-Abl.

The v-Abl oncoprotein is a nonreceptor tyrosine kinase encoded by the Abelson murine leukemia virus (A-MuLV), which causes pro-/pre-B cell lymphomas in mice (1, 2). v-Abl provides an excellent system for dissecting the individual molecular events required for malignant transformation. v-Abl constitutively activates multiple signaling pathways, including the Ras, Rac, phosphatidylinositol 3-kinase, and STAT pathways, all of which contribute to its transforming potential (3, 4). However, the clonal/oligoclonal nature of tumors induced by A-MuLV (5) implies that despite being a potent oncogene, v-Abl by itself is not sufficient to cause transformation. Additional genetic alterations are believed to be necessary for v-Abl-mediated transformation (6).

Previous studies have shown that loss of p53 facilitates v-Abl-mediated transformation in B cells (7, 8). In primary mouse embryonic fibroblasts, loss of p53 is essential for v-Abl-mediated transformation, because wild type MEFs undergo growth arrest in response to v-Abl (9). However, only a small proportion of A-MuLV-infected p53−/− MEFs are eventually transformed, suggesting that even in a p53 null background cells that become transformed have undergone additional mutations that allow transformation. Moreover, loss of p53 function in nonpermissive immortal fibroblasts failed to relieve v-Abl toxicity, suggesting a p53-independent mechanism of v-Abl toxicity in these cells (9).

p53−/− MEFs can be transformed by v-Abl but, unlike primary pre-B cells, do not depend on v-Abl for extended growth in culture. Furthermore, loss of p53 causes genetic instability (10–12), which would be predicted to accelerate additional genetic alterations that might enhance v-Abl-dependent transformation. We exploited these characteristics of p53−/− MEFs to create an experimental system for identification of genetic changes associated with v-Abl-dependent transformation. A comparison of p53−/− MEFs transiently infected with v-Abl retrovirus and v-Abl-transformed MEF clones allowed us to distinguish the initial response of MEFs to v-Abl, from transformation-associated changes. In addition, comparing untransformed p53−/− MEFs to v-Abl-transformed p53−/− MEF clones treated with the Abl kinase inhibitor STI 571 (13, 14) allowed us to identify additional genetic changes that occur during the process of v-Abl transformation.

Our studies reveal that mRNA encoding an anti-apoptotic member of the Bcl-2 family, Bcl-xL, is elevated in a v-Abl kinase-independent manner in three of the five v-Abl-transformed MEF clones that were studied. In addition, overexpression of c-Myc mRNA, either through gene amplification or through enhanced signaling via STAT3, was identified as a secondary event in five of six clones tested. Overexpression of c-Myc provided v-Abl-transformed MEF clones with a growth advantage, which was evident in their decreased doubling times and elevation of telomerase activity. These observations support a role for the c-myc proto-oncogene in stabilizing the malignant phenotype of v-Abl-transformed MEFs.

EXPERIMENTAL PROCEDURES

Retroviral Vectors—The Vxy-IRES-puro retroviral vector was a gift from Lou Staudt (NCI, National Institutes of Health, Bethesda, MD). The coding sequence for p160 v-Abl was cloned into the EcoRI site of Vxy-IRES-puro by blunt end ligation, thus creating a bi-cistronic retrovirus expressing a temperature-sensitive v-Abl kinase and green fluorescent protein was a kind gift from Dr. Naomi Rosenberg (Tufts University School of Medicine, Boston, MA).

Production of Retrovirus from 293T Cells and Infection of MEFs—293T cells were transfected by the calcium phosphate precipitation method with 20 μg of retrovirus construct, 15 μg of VSVG packaging DNA encoding gag and pol genes, and 15 μg of pMDG-VSVG pseudotyped envelope protein following the Nolan lab protocol (www. stanford.edu/group/nolan/protocols/pro_helper_dep.html). Pseudotyped virus was concentrated by centrifugation at 80,000 × g at 4 °C for 90 min, and viral pellets were resuspended in 30 μl of Hanks’ balanced salt solution (Invitrogen) by gentle agitation at 4 °C for a minimum of 1 h. MEFs were plated at a density of 1 × 105 cells/well in a 6-well plate. On the day of infection, 2 μl of puro at 5 μg/ml polybrene and 50 μl of concentrated virus were added to 2 ml of growth medium in each well, and the
plates were spun at 800 × g for 60–90 min at room temperature. The plates were then incubated overnight at 32 °C. The virus-containing medium was aspirated and replaced with fresh growth medium, and the cells were transferred to a 37 °C incubator. p53−/− MEFs infected with puro viruses were expanded in medium for 2 days after infection and selected with puromycin at a concentration of 1.4 μg/ml for 3 days. RNA extracts were prepared from puromycin-resistant cells.

Cell Culture—MEFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 20 μg/ml of gentamicin sulfate.

MEF clones 9, 19, 46, and 12R transformed with wild type v-Abl were generated by infecting p53−/− MEFs with wild type v-Abl virus obtained from a producer line that makes both v-Abl and Moloney helper viruses (9). MEF clone ts60 was generated by infecting from a producer line that makes both v-Abl and Moloney helper viruses. Two days post-infection, MEFs were plated in medium containing 1.4 μg/ml of puromycin. Total RNA was isolated after 3 days of puromycin selection and analyzed by RPA. There was no difference in the mRNA levels of Bcl-xL (anti-apoptotic), Bak (pro-apoptotic), and Bax (pro-apoptotic) between v-Abl-transformed MEF clones (Fig. 1). The levels of Bcl-xL (anti-apoptotic) and Bak (pro-apoptotic) mRNA were too low to be quantified (data not shown). Thus, in MEFs, acute expression of v-Abl does not alter steady-state transcripts of Bcl-2 family genes, Bcl-xL, Bak, and Bax.

To determine whether the process of v-Abl transformation selects cells with altered steady-state levels of Bcl-2 family transcripts, a similar RPA was performed on individual p53−/− MEF clones transformed by v-Abl. Clones 1, 9, 19, 46, and 12R were compared with untransformed p53−/− MEFs. v-Abl-transformed p53−/− MEF clone 1 has been described previously (9). Clones 9, 19, 46, and 12R were freshly isolated and analyzed under “Experimental Procedures.” Total RNA from untransformed p53−/− MEFs and from the v-Abl-transformed MEF clones was analyzed for steady-state mRNA levels of Bcl-2 family members Bcl-xL, Bak, and Bax.

Steady-state mRNA and Protein Levels of Anti-apoptotic Protein Bcl-xL and c-Myc Induction during v-Abl Transformation

Steady-state mRNA levels of Bcl-2 family members were analyzed by RPA in p53−/− MEFs acutely infected with a v-Abl/puroR bicistronic retrovirus or a control puroR retrovirus. Two days post-infection, MEFs were plated in medium containing 1.4 μg/ml of puromycin. The bands were quantified using ImageQuant software and normalized to ribosomal protein L32.

RESULTS

Steady-state mRNA and Protein Levels of Anti-apoptotic Protein Bcl-xL Are Elevated in Three of Five p53−/− MEF Clones Transformed by v-Abl—Decreased apoptosis is often important in transformation (21–23), and Bcl-2 family proteins are critical regulators of apoptosis (24, 25). We therefore determined whether and how expression of v-Abl affects expression of Bcl-2 family members in our experimental system.

Steady-state mRNA levels of Bcl-2 family members were compared with untransformed MEFs (data not shown). Bcl-2 and Bad mRNA levels were too low to be quantified. In clones 1 and 12R, Bcl-xL mRNA levels were not significantly different from those of untransformed MEFs (data not shown). However, clones 9, 19, and 46 had a significant increase in Bcl-xL mRNA (2–4-fold; Fig. 2A). A corresponding increase was also observed in Bcl-xL protein (Fig. 2B). To explore the increase in Bcl-xL mRNA further, we determined whether it depended on v-Abl kinase
activity by treating clones 9, 19, and 46 with STI571 (Fig. 3A). v-Abl kinase activity was completely abolished following STI 571 treatment in all clones, as determined by v-Abl autophosphorylation (Fig. 3B). Interestingly, the increased Bcl-xL mRNA was not reduced following treatment of clones 9, 19, and 46 with STI 571 (Fig. 3A), establishing that the elevated Bcl-xL mRNA levels are not dependent on v-Abl kinase activity. Thus, in clones 9, 19, and 46, the v-Abl transformation process has selected for MEFs with elevated levels of mRNA encoding the anti-apoptotic protein Bcl-xL.

Elevated Levels of c-Myc mRNA and Protein Are Found in Clones of p53−/− MEFs Transformed by v-Abl—Induction of c-Myc mRNA by v-Abl is one of several mitogenic events required for v-Abl-dependent transformation (3, 26). v-Abl induces mRNA for c-myc and a variety of other E2F-dependent genes by initiating a signaling pathway that results in phosphorylation of Rb family proteins and subsequent activation of E2F family proteins (27). We determined how expression of various E2F-dependent genes was affected by acute expression of v-Abl in p53−/− MEFs and in the p53−/− MEF clones transformed by v-Abl.

Steady-state mRNA levels of E2F-dependent genes c-myc, p107, and p19ARF were compared by RPA in p53−/− MEFs, in p53−/− MEFs acutely infected with a v-Abl retrovirus, and in v-Abl-transformed MEF clone 1. Steady-state mRNA for p107, c-Myc, and p19ARF were induced 2–3-fold in p53−/− MEFs acutely expressing v-Abl (Fig. 4a, lanes 2, 5, and 8). A similar increase in p107 and p19ARF mRNA was observed in transformed clone 1 (Fig. 4a, lanes 3 and 6). These increases are consistent with previous work showing induction of E2F-dependent genes by v-Abl (16, 27). However, c-Myc mRNA levels in clone 1 were 30-fold higher than in untreated MEFs and 10-fold higher than in MEFs acutely expressing v-Abl (Fig. 4a, lanes 7–9).

The c-Myc mRNA levels were explored further. Aliquots of clone 1 frozen prior to extensive growth in culture were unavailable. However, we had samples of other v-Abl-transformed p53−/− MEF clones that were frozen immediately after soft agar cloning and also following growth in culture for approximately 6 months (see “Experimental Procedures” for details). We performed RPA analyses for c-Myc, p107, and p19ARF mRNA with total RNA from clones 9, 19, 46, and 12R, comparing freshly isolated clones to those that had been grown in culture for 6 months. Similar to our observations for clone 1, a 2–4-fold increase in p107 and p19ARF mRNA was observed in all freshly isolated clones, and there was no significant change in mRNA levels of p19ARF and p107 in clones 9, 19, 46, and 12R following growth in culture (Fig. 4b and Table I). c-Myc mRNA was induced ~2-fold in clones 9, 46, and 12R and 4-fold in clone 19 in the early isolates. However, after 6 months in culture, the c-Myc mRNA levels had increased an additional 2-fold in clones 9, 19, and 12R, whereas a 6-fold increase was observed in clone 46 (Fig. 4b and Table I). The increase in c-Myc mRNA levels was also reflected in increased c-Myc protein levels in clones 9, 19, 46, and 12R (Fig. 5). However, the fold increases in c-Myc protein did not correspond precisely with mRNA levels in every case. This may be due to additional mechanisms that regulate c-Myc protein levels.

Treatment of clones 1, 19, and 12R with STI 571 reduced c-Myc mRNA to levels close to those seen in untransformed p53−/− MEFs (Fig. 6, a, lane 5, and b, lanes 4 and 6). However,
Fig. 4. Steady-state mRNA levels of E2F-dependent genes in untransformed p53−/− MEFs, p53−/− MEFs transiently infected with v-Abl retrovirus, and v-Abl-transformed p53−/− MEF cell lines. a, equal amounts of total RNA from untransformed p53−/− MEFs (lanes 1, 4, and 7), p53−/− MEFs transiently infected with v-Abl retrovirus (lanes 2, 5, and 8), and from v-Abl-transformed MEF clone 1 (lanes 3, 6, and 9) were used in a RPA to determine mRNA levels of E2F dependent genes, p107 (upper panel, lanes 1–3), p19ARF (upper panel, lanes 4–6), and c-myc (upper panel, lanes 7–9). The bands were quantified using ImageQuant software and normalized to actin controls (lower panel, lanes 1–9). b, equal amounts of total RNA from untransformed p53−/− MEFs (lane 1) and v-Abl-transformed clones 9, 19, and 12R, either freshly isolated (lanes 2–5) or after 6 months in culture (lanes 6–9), were tested in a RPA for c-Myc, p19ARF, and p107 mRNA levels. The bands were quantified using ImageQuant software and normalized to actin controls.

Table I

| Clone           | c-Myc (mean ± S.D.) | p19ARF (mean ± S.D.) | p107 (mean ± S.D.) |
|-----------------|---------------------|----------------------|-------------------|
| Clone 9, fresh isolate | 1.9 ± 0.2           | 2.9 ± 0.4            | 1.6 ± 0.3         |
| Clone 9, 6-month culture | 3.8 ± 0.2           | 4.2 ± 0.2            | 1.8 ± 0.4         |
| Clone 19, fresh isolate    | 4.4 ± 0.3           | 4.1 ± 0.2            | 1.4 ± 0.2         |
| Clone 19, 6-month culture | 7.3 ± 0.8           | 4.5 ± 0.3            | 1.8 ± 0.2         |
| Clone 46, fresh isolate    | 2.1 ± 0.1           | 2.8 ± 0.2            | 1.6 ± 0.4         |
| Clone 46, 6-month culture | 12.6 ± 0.4          | 4.3 ± 0.3            | 1.9 ± 0.1         |
| Clone 12R, fresh isolate   | 2.6 ± 0.04          | 2.1 ± 0.2            | 1.4 ± 0.2         |
| Clone 12R, 6-month culture | 4.7 ± 0.4           | 2.8 ± 0.4            | 1.8 ± 0.2         |
| Clone 1           | 17.6 ± 1.05         | 3.1 ± 0.07           | 2.6 ± 0.4         |

Changes in expression of E2F-dependent genes during cell culture in v-Abl transformed MEF clones 9, 19, 46, and 12R

Total RNA from untransformed p53−/− MEFs and v-Abl transformed clones 9, 19, 46, and 12R, either freshly isolated or after 6 months in culture, were tested in a RPA for c-Myc, p19ARF, and p107 mRNA levels. The bands were quantified using ImageQuant software and normalized to actin controls. The results from four independent experiments are represented as fold change (means ± S.D.) as compared with untransformed p53−/− MEFs.

Fig. 5. c-Myc protein is induced in v-Abl-transformed MEF clones 9, 19, 46, and 12R. Whole cell extracts from untransformed p53−/− MEFs (lane 1 and 10), v-Abl-transformed MEF clones 9, 19, 46, and 12R, either freshly isolated (lanes 2–5) or after 6 months in culture (lanes 6–9) and clone 1 (lane 11) were analyzed by Western blot using anti-c-Myc antibodies. The bands were quantified using ImageQuant software and normalized to actin controls.

treatment of clones 46 and ts60 (a p53−/− MEF clone transformed by a temperature-sensitive form of v-Abl kinase) with STI 571 did not reduce c-Myc mRNA levels to those seen in untransformed p53−/− MEFs (Fig. 6c, lanes 4 and 6), indicating that c-Myc mRNA induction was not completely dependent on v-Abl kinase activity in these clones. Following treatment with STI 571 (as described under “Experimental Procedures”), v-Abl kinase was inactivated in all clones (data not shown).

Thus, steady-state mRNA encoding c-Myc is elevated in five of six v-Abl-transformed p53−/− MEF clones (clones 1, 12R, 19, 46, and ts60 but not clone 9), when compared with MEFs acutely expressing v-Abl. In clones 12R, 19, and 46, we documented that the increase in c-Myc mRNA occurred during growth in culture. These increases were specific to c-Myc and were not observed for other E2F-dependent genes, p107 and p19ARF. The induction of c-Myc mRNA was dependent on v-Abl in clones 1, 12R, and 19, whereas it was only partially dependent on v-Abl in clones 46 and ts60.

c-myc Gene Amplification Explains Elevated mRNA Levels in Four v-Abl-transformed p53−/− MEF Clones—What mechanism might account for the observed elevation in c-Myc mRNA in the v-Abl-transformed p53−/− MEF clones following growth in culture? Taking into consideration that elevated mRNA levels were wholly or partially dependent on v-Abl but were not
Enhanced STAT3 Activation Occurs in Two v-Abl-transformed p53−/− MEF Clones—The third model is based on the observation that v-Abl activates c-myc transcription not only by activating E2F family proteins but also by activating STAT3, which binds an adjacent site in the c-myc promoter (28). STAT3 activation was analyzed in clones 9, 19, 46, and 12R. Whole cell extracts from serum-starved cells were analyzed by Western blotting using anti-phospho-STAT3 antibodies. To control for equal loading, the blots were stripped and reprobed with anti-STAT3 antibodies. As shown in Fig. 9, clones 9 and 46 had modest increases in phosphorylated STAT3 relative to untransformed p53−/− MEFs, which likely reflects constitutive activation by v-Abl. However, clone 19 and, to a lesser extent, clone 12R, contained significantly more activated STAT3 than clones 9 and 46. Thus, increased activation of STAT3 is involved in elevated c-Myc mRNA in clones 19 and 12R.

Elevated c-Myc Expression Correlates with Increased Growth of v-Abl-transformed MEFs—We wished to determine whether elevated c-Myc mRNA in the v-Abl-transformed p53−/− MEF clones was accompanied by known downstream effects of c-Myc. The doubling times of v-Abl-transformed MEF clones were compared with untransformed p53−/− MEFs (Fig. 10a). Clone 1, which contains ~30 copies of c-myc and has the highest levels of c-Myc mRNA, had a doubling time of 8 h. Clone 19, which displays no gene amplification but a significant increase in phosphorylated STAT3 and an intermediate level of c-Myc mRNA, had a doubling time of 14 h. Clone 9, which exhibits the lowest levels of c-Myc mRNA, had a doubling time of 22 h. Thus, there is a strong correlation between the doubling time of the different clones and their relative c-Myc mRNA levels, suggesting that elevated c-Myc does indeed provide a proliferative advantage.

Transcription of telomerase reverse transcriptase, the catalytic subunit of telomerase, is induced by c-Myc (29). Activation of telomerase allows transformed cells to bypass crisis caused by erosion of telomeres (30, 31). Therefore, levels of telomerase in whole cell extracts of untransformed p53−/− MEFs and v-Abl-transformed MEF clone 1 were measured by the telomeric repeat amplification protocol assay. Telomerase activity was significantly increased in clone 1 compared with untransformed p53−/− MEFs (Fig. 10b, lane 2b). Moreover, treatment of clone 1 with STI 571 caused a reduction of telomerase activity to levels seen in untransformed MEFs, concomitant with the reduction in c-Myc mRNA levels (Fig. 10b, lane 3b). Thus, elevation of c-Myc is associated with shorter doubling times and higher telomerase levels.

observed in other E2F-dependent genes and thus were unlikely to depend on levels of activated E2F, we considered three possible mechanisms: 1) mutations in the E2F/STAT3 binding site of the c-myc promoter resulting in an elevated response to v-Abl signaling via E2F or STAT3, 2) c-myc gene amplification, or 3) increased responsiveness of the STAT3 pathway to v-Abl. When the E2F/STAT3-binding sites in the c-myc promoters of clones 1, 9, 19, 46, and 12R were sequenced, all of the clones had the wild type sequence (data not shown), ruling out the first model.

To address the second possibility, genomic DNA was analyzed by Southern blotting. As shown in Fig. 7a, clone 1 contained ~30 copies of the c-myc gene. Southern analysis was also performed on clones 9, 19, 46, and 12R, using DNA from both freshly isolated clones and clones grown in culture for 6 months. No c-myc gene amplification was evident in any of the freshly isolated clones. However, clones 12R and 46 acquired c-myc gene amplification (approximately three copies in clone 12R and 32 copies in clone 46) after 6 months in culture (Fig. 7b, lanes 8 and 9). c-myc gene amplification during growth in culture was also observed in v-Abl-transformed clone, ts60. Southern blot analysis of ts60 at various time points during culture indicated that c-myc gene amplification was acquired after 2 months in cell culture (Fig. 7c, lanes 4 and 5). Southern analysis of an additional six freshly isolated v-Abl-transformed p53−/− MEF clones did not reveal c-myc gene amplification (Fig. 8), further confirming that c-myc gene amplification was a secondary event. Thus, gene amplification during growth in culture appears to account for elevated c-Myc mRNA levels in clones 1, 12R, 46, and ts60.

FIG. 6. c-Myc overexpression is wholly or partially dependent on v-Abl kinase activity in MEF clones 1, 9, 12R, 46, and ts60. a, equal amounts of total RNA from untransformed p53−/− MEFs grown in control medium (lane 1) or in medium containing 0.4 μM STI 571 (lane 2), p53−/− MEFs transiently infected with v-Abl retrovirus (lane 3) and clone 1 cells grown in control medium (lane 4) or in medium containing 0.4 μM STI 571 (lane 5) were tested in a RPA for c-Myc mRNA levels (upper panel). The bands were quantified using ImageQuant software and normalized to actin controls (lower panel). b, equal amounts of total RNA from untransformed p53−/− MEFs (lanes 1 and 2), clones 19 (lanes 3 and 4), and 12R (lanes 5 and 6) grown in control medium (lanes 1, 3, and 5) or in medium containing 0.4 μM STI 571 (lanes 2, 4, and 6) were tested in a RPA for c-Myc mRNA levels (upper panel). The bands were quantified using ImageQuant software and normalized to actin controls (lower panel). c, equal amounts of total RNA from untransformed p53−/− MEFs (lanes 1 and 2), clones 46 (lanes 3 and 4), and ts60 (lanes 5 and 6) grown in control medium (lanes 1, 3, and 5) or in medium containing 0.4 μM STI 571 (lanes 2, 4, and 6) were tested in a RPA for c-Myc mRNA levels (upper panel). The bands were quantified using ImageQuant software and normalized to actin controls (lower panel).
DISCUSSION

Malignant transformation is understood to be a multi-step process, the components of which include constitutive mitogenic signals, evasion of apoptotic and anti-growth signals, stable telomere maintenance, and, for solid tumors, acquisition of angiogenesis and tissue invasion capabilities (32). Although A-MuLV is an acute transforming virus providing constitutive

Fig. 7. c-myc gene amplification is a secondary event in v-Abl-transformed p53−/− MEF clones 1, 12R, 46, and ts60. a, genomic DNA from untransformed p53−/− MEFs (lane 1) and clone 1 (lane 2) was analyzed by Southern blot using a c-myc probe (upper panel). The actin probe (lower panel) was used as a loading control. b, genomic DNA from untransformed p53−/− MEFs (lane 1) and v-Abl-transformed MEF clones 9, 19, 46, and 12R, either freshly isolated (lanes 2–5) or those maintained in culture for 6 months (lanes 6–9), was analyzed by Southern blot using c-myc (upper panel) and actin (lower panel) probes. c, genomic DNA from untransformed p53−/− MEFs (lane 1) and from v-Abl-transformed MEF clone ts60 (lanes 2–5) harvested at indicated time points during culture was analyzed by Southern blot using c-myc (upper panel) and actin (lower panel) probes.

Fig. 8. c-myc gene is not amplified in freshly isolated v-Abl-transformed p53−/− MEF clones 5, 6, 7, 25, 33, and 45. Genomic DNA from untransformed p53−/− MEFs (lane 1) and v-Abl-transformed p53−/− MEF clones 5, 6, 7, 25, 33, and 45 (lanes 2–7) was analyzed by Southern blot using c-myc (upper panel) and actin (lower panel) probes.

Fig. 9. Enhanced v-Abl-mediated signaling through STAT3 contributes to elevated c-Myc mRNA levels in clone 19, in the absence of c-myc gene amplification. Whole cell extracts prepared from serum-starved untransformed p53−/− MEFs (lane 1) and v-Abl-transformed MEF clones 9, 19, 46 and 12R (lanes 2–5) were analyzed by Western blot using anti-phosho-STAT3 and anti-STAT3 antibodies. The bands were quantified using ImageQuant software and normalized to total STAT3 levels.
of stably transformed clones (7, 34).

Inhibition of p53-dependent growth arrest or apoptotic pathways has been identified as one component of v-Abl transformation (7–9). Additional genetic alterations, although implied by the oligoclonal transformation of p53−/− cells, have not been identified for v-Abl-dependent transformation. Here we present evidence identifying elevated expression of mRNAs encoding the anti-apoptotic protein Bcl-xL and the oncoprotein c-Myc as genetic alterations that frequently accompany v-Abl transformation of p53−/− MEFs. We also show that elevated c-Myc levels are accompanied by a growth advantage and increased telomerase activity in v-Abl-transformed clones.

Bcl-xL—Bcl-xL mRNA and protein was found to be elevated in three of five v-Abl-transformed p53−/− MEF clones tested. Given the anti-apoptotic activity of Bcl-xL (24, 35) and the fact that elevated Bcl-xL expression has been observed in other murine myeloid and T cell malignancies (36), it is reasonable to suggest that this alteration facilitates transformation. Increased levels of Bcl-xL mRNA could result from a mutation in a regulatory element of the bcl-xL gene itself or from a mutation in a component of regulatory pathways that control either the transcription or the stability of Bcl-xL mRNA. Although previous studies provide evidence for v-Abl-dependent induction of Bcl-xL mRNA in mast cells and pre-B cells (37, 38), the increase in Bcl-xL mRNA in the MEF clones was independent of v-Abl kinase activity (Fig. 3). This may reflect differences between fibroblasts and hematopoietic lineages. It is consistent with the idea that the mutation(s) causing increased Bcl-xL mRNA may have been present and growth-selected upon v-Abl expression; alternatively the mutation(s) may have occurred and been selected following v-Abl expression.

c-Myc—Deregulated expression of c-Myc is well known to be oncogenic in many settings (39, 40) and a requirement for induction of c-myc transcription has been well established for transformation by both v-Abl and BCR-ABL (26). v-Abl activates E2F-dependent genes, thus providing mitogenic signals that drive G1/S cell cycle progression in fibroblasts (3, 16, 27). The data presented here confirm that steady-state mRNA levels of E2F-dependent genes c-myc, p19ARF, and p107 are induced 2–4-fold by v-Abl-both in MEFs transiently infected with v-Abl and in freshly isolated v-Abl-transformed MEF clones.

However, our data show that in addition to v-Abl-dependent induction of c-myc transcription, events that occur later in the transformation process often elevate c-Myc mRNA levels further and are likely to be important for v-Abl transformation. Clones 12R, 19, and 46 acquired elevated c-Myc mRNA levels (Fig. 46 and Table I) during culture. Most significantly, c-Myc mRNA levels correlated with increased doubling times and elevated levels of telomerase activity (Fig. 10), providing strong evidence that this later increase plays a role in transformation.

The increased mRNA was specific for c-myc and was not found in other E2F-dependent genes, such as p107 or p19ARF. Furthermore, c-myc transcriptional induction was completely dependent on v-Abl in clones 1, 12R, and 19 but only partially dependent on v-Abl in clones 46 and ts60. We have identified two different genetic mechanisms responsible for elevated c-myc mRNA in these clones; clones 12R, 46, and ts60 exhibited c-myc gene amplification, whereas clone 19 displayed enhanced STAT3 activity.

Consistent with our findings, the loss of p53 is known to cause genomic instability (10–12), and c-Myc overexpression and gene amplification have been observed in cells from various organs of 4–6-week old p53−/− mice (12). Furthermore, amplification of c-myc has been observed previously in v-Abl-transformed NIH3T3 cell lines (41, 42). It is likely that genomic instability caused by the loss of p53 contributed to the large
fraction of clones that acquired c-myc gene amplification. v-Abl activates STATs (43–45), and STAT3 is known to activate c-myc transcription (28). It therefore seems likely that the increase in STAT3 activity that we observed in clone 19 is mechanistically responsible for the elevated c-Myc mRNA levels observed in this clone. The genetic alteration responsible for elevated STAT3 phosphorylation is not known but could be caused by changes in Jak activity, protein phosphatase activity, or the STAT3 protein itself.

In summary, by using an experimental system that is able to reveal genetic changes associated with v-Abl transformation, we have identified increases in Bel-xL and c-Myc mRNA as changes that occur during the process of v-Abl transformation. Our findings underline the importance of anti-apoptotic and hyperproliferative changes during transformation initiated by constitutive expression of the v-Abl kinase.

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