Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein

(Abelson murine leukemia virus/Philadelphia chromosome/tyrosine kinase)

GEORGE Q. DALEY AND DAVID BALTIMORE

Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142; and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by David Baltimore, September 6, 1988

ABSTRACT The P210bcr/abl protein is associated with virtually every case of human chronic myelogenous leukemia. Unlike the related P160p210bcr/abl oncogene product of Abelson murine leukemia virus, P210bcr/abl does not transform NIH 3T3 fibroblasts. To assess whether P210bcr/abl might transform hematopoietic cell types, retroviral constructs encoding P210bcr/abl were used to infect the bone marrow-derived inter leukin 3-dependent Ba/F3 cell line. As for P160p210bcr/abl, cell lines expressing P210bcr/abl were growth factor independent and tumorigenic in nude mice. No evidence for autocrine production of interleukin 3 by factor-independent cell lines was found. These experiments establish that P210bcr/abl can transform hematopoietic cell types to tumorigenicity.

The P210bcr/abl protein derives from a hybrid gene created by the chromosomal translocation that generates the Philadelphia chromosome, a cytogenetic abnormality which characterizes human chronic myelogenous leukemia (CML) cells (1). The CML-specific P210 protein shares structural and enzymatic properties with the v-abl protein of the Abelson murine leukemia virus (A-MuLV). The gene for P210 and v-abl arise by substitution of the c-abl5 sequence encoding the N-terminal region with bcr (2) and helper-virus-derived gag sequences (3), respectively. Both proteins exhibit elevated tyrosine-specific protein kinase activity (4). The v-abl protein is responsible for the induction of acute lymphosarcomas in susceptible murine hosts infected with A-MuLV (5). The role of P210 in the etiology of human CML remains to be defined.

The v-abl protein can transform a variety of cell types. It efficiently transforms NIH 3T3 fibroblasts in vitro (6) and pre-B-lymphoid cells both in vitro and in vivo (7) and is able to relieve the growth-factor dependence of several hematopoietic cell types, including T-cell lines dependent on interleukin 2 (8), and various interleukin 3 (IL-3)-dependent lymphoid and myeloid cell lines (9-13). Unlike v-abl, P210 does not transform NIH 3T3 fibroblasts (14). In this study, we demonstrate that P210 resembles v-abl in its ability to transform the IL-3-dependent hematopoietic cell line Ba/F3 (15) to factor independence and tumorigenicity. These experiments demonstrate the oncogenic potential of P210bcr/abl.

MATERIALS AND METHODS

Plasmid Constructs, Viral Stocks, and Cell Culture Conditions. A 4.7-kilobase (kb) fragment containing coding sequence for the full-length P160 isolate of v-abl (16) was cloned into the BamHI cloning site of the pWE vector (B. Guido and R. C. Mulligan, Whitehead Institute) by using BamHI link-
dispensed in triplicate into 96-well plates (2.5 × 10⁴ cells per well). Test supernatants were added in appropriate dilution to the plates, which were incubated for 24 hr at 37°C. 3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added to each well, and the cells were further incubated for 5 hr. The absorbance was measured on a Dynatech model MR580 plate reader at a wavelength of 570 nm. A neutralizing polyclonal anti-IL-3 IgG was a gift from P. Vassalli (University of Geneva), and a control polyclonal IgG specific for the epidermal growth factor receptor was a gift of Y. Yarden (Whitehead Institute). IgG was preincubated with conditioned medium for 1 hr prior to addition to the proliferation assay.

Nucleic Acid and Protein Analysis. High molecular weight genomic DNA was isolated from relevant cell lines, digested with the enzyme Xba I, electrophoresed through a 0.7% agarose gel, transferred to a nylon membrane, and probed with a fragment of the G418-resistance (Neo) gene labeled to high specific activity (>10⁹ cpm/μg). Hybridization and wash conditions were of high stringency. Immunoprecipitation analysis of abl proteins was as described (22).

Assay of Tumorigenicity in Nude Mice. Cells for assay of tumorigenicity were washed with serum-free medium and resuspended in Hanks’ balanced saline solution. About 2 × 10⁴ cells were injected subcutaneously into young (<12 week old) nude mice (BALB/c- AnNCr-nu) from the National Cancer Institute. Mice received 500 rads (1 rad = 0.01 Gy) of γ-irradiation 24 hr prior to cell challenge. Mice were observed for 2–3 months for signs of palpable or visible tumor at the site of injection. Tumorigenic cell lines gave rise to a visible pea-sized mass with a short latency after injection which was nonregressing and malignant. Nontumorigenic cell lines showed no evidence of tumor for up to 3 months after injection.

RESULTS

Constructs for expression of abl protein variants were made by using the pWE vector (Fig. 1). The pWE vector carries a dominant coselectable antibiotic-resistance marker expressed from the promoter element of the Moloney virus LTR. The cloned abl sequence is expressed from an internal promoter derived from the chicken β-actin gene. Titers of retroviral producer cell supernatants were determined by resistance of infected NIH 3T3 fibroblasts to the antibiotic G418 and were comparable for all constructs (10⁵ colony-forming units/ml). IL-3-dependent Ba/F3 cells were infected with retroviral-producer cell culture supernatants and grown for 48 hr in the presence of WEHI-3B conditioned medium prior to selection (Fig. 2). Primary selection for viral infection was carried out in medium supplemented with both WEHI-3B conditioned medium and G418 at 2 mg/ml. For all infected cell lines, G418-resistant populations arose after 7–10 days of selection, suggesting that >0.1% of cells had been infected. To select for IL-3 independence, G418-resistant populations were washed extensively with PBS and cultured in medium lacking a source of IL-3. Cell populations infected with the pWE210 virus (encoding P210βcr/abl) or the pWEGab virus (encoding P160mg/V-abl) gave rise to populations of IL-3–independent cells after 5–10 days, suggesting that between 0.1% and 5% of cells survived selection. A G418-resistant population of cells infected with the pWE virus alone or uninfected cells did not survive selection in medium lacking a source of IL-3, demonstrating that abl sequences are required to generate IL-3 independence (Table 1).

Uninfected Ba/F3 cells proliferate maximally in the presence of 3–10% (vol/vol) conditioned medium from WEHI-3B cells, and their proliferation declines upon dilution of the conditioned medium (Fig. 3). The proliferation profile for Ba/F3 cells infected with the pWE virus alone is similar to that for the uninfected Ba/F3 cell line (Fig. 3A). Cells infected with viruses that encode P210βcr/abl or P160mg/V-abl and selected for growth in the absence of exogenous IL-3 proliferate in a factor-independent manner, without regard to the concentration of conditioned medium from WEHI-3B cells. Their growth rate is equivalent to the parental Ba/F3 cell line growing in medium supplemented with IL-3 (unpublished data). Conditioned medium from the IL-3–independent cell lines infected with either the pWE210 or pWEGab viruses will not support the proliferation of uninfected Ba/F3 cells. This suggests that the pWE210- and pWEGab-infected cells do not liberate a growth factor into the medium that can support their growth by autocrine stimulation. Analysis of total RNA from the IL-3–independent cell lines failed to detect expression of IL-3 mRNA, although it could readily be detected in WEHI-3B cells (unpublished data). Fig. 3B shows the results of experiments with a specific antibody to IL-3 capable of neutralizing its growth-promoting activity. IgG directed at an irrelevant antigen (the epidermal growth factor receptor) failed to alter the proliferation profile for uninfected Ba/F3 cells. IgG specific for IL-3 inhibited the proliferative activity of WEHI-3B conditioned medium for uninfected Ba/F3 cells. The IL-3–independent Ba/F3 cells infected with either the pWE210 or pWEGab viruses were assayed in medium lacking WEHI-3B conditioned medium in the presence of neutralizing anti-IL-3 IgG. The proliferation of the pWE210- and pWEGab-infected cells was unaffected by the presence of the neutralizing IgG.

![Fig. 1. Retroviral constructs.](https://example.com/fig1.png)

The pWE vector is a derivative of the Moloney murine leukemia virus (B. Guild and R. C. Mulligan, Whitehead Institute). The Neo gene, encoding resistance to the antibiotic G418, is expressed from the 5’ LTR. An internal promoter from the chicken β-actin gene directs the expression of cloned cDNA inserted into the unique BamHI cloning site. SV and pBR represent sequences for the simian virus 40 and pBR322 plasmid origins of replication. For pWE210, the cDNA for the full-length coding region of the P210βcr/abl protein (cDNA 172/215, ref. 14) was cloned into the pWE vector by using Bcl I linkers. For pWEGab, sequences encoding the P160mg/V-abl isolate of A-MuLV (16) were cloned into the pWE vector by using BamHI linkers.
To establish whether infected cells could produce tumors in nude mice, the number of mice that developed large, nonregressing tumors at the site of subcutaneous injection of cells over the course of the experiment is shown. To determine whether conditioned medium could support Ba/F3 cell proliferation, conditioned medium was harvested from helper-virus-free cell lines at high density and passed through a 0.45-μm filter. To establish the capacity to develop Ba/F3 cell lines under conditions of primary selection (G418 resistance) or secondary selection (IL-3 independence), + indicates establishment of continuously proliferating cell line and − indicates cell line will not grow under specified conditions. The association of the Philadelphia chromosome with the development of chronic myelogenous leukemia (CML) is well documented. CML is characterized by the formation of a unique fusion protein, P210bcr/abl, which results from the translocation of the bcr and abl genes at chromosome 22 and 9, respectively. The Philadelphia chromosome is also associated with the development of Philadelphia chromosome-negative CML, which is characterized by the formation of a cytoplasmic protein, P160sag/v-abl, that is not detectable in normal cells.

Table 1. Properties of infected Ba/F3 cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein</th>
<th>Growth of Ba/F3 cells in selective medium after viral infection</th>
<th>Tumors in nude mice</th>
<th>Ability of conditioned medium to support Ba/F3 cell proliferation</th>
<th>Properties of rescued virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Primary, IL-3/ + G418</td>
<td>Secondary, IL-3/ + G418</td>
<td></td>
<td>Passes Neo gene</td>
</tr>
<tr>
<td>Mock</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
<td>0/8</td>
</tr>
<tr>
<td>pWE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0/7</td>
</tr>
<tr>
<td>pWEgab P160Gab/v-abl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9/9</td>
</tr>
<tr>
<td>pWE210 P210bcr/abl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>12/12</td>
</tr>
</tbody>
</table>

For the capacity to establish Ba/F3 cell lines under conditions of primary selection (G418 resistance) or secondary selection (IL-3 independence), + indicates establishment of continuously proliferating cell line and − indicates cell line will not grow under specified conditions. To establish whether infected cells could produce tumors in nude mice, the number of mice that developed large, nonregressing tumors at the site of subcutaneous injection of cells was shown. To determine whether conditioned medium could support Ba/F3 cell proliferation, conditioned medium was harvested from helper-virus-free cell lines at high density and passed through a 0.45-μm filter. To establish the capacity to develop Ba/F3 cell lines under conditions of primary selection (G418 resistance) or secondary selection (IL-3 independence), + indicates establishment of continuously proliferating cell line and − indicates cell line will not grow under specified conditions. To establish whether infected cells could produce tumors in nude mice, the number of mice that developed large, nonregressing tumors at the site of subcutaneous injection of cells over the course of the experiment is shown. To determine whether conditioned medium could support Ba/F3 cell proliferation, conditioned medium was harvested from helper-virus-free cell lines at high density and passed through a 0.45-μm filter. To establish the capacity to develop Ba/F3 cell lines under conditions of primary selection (G418 resistance) or secondary selection (IL-3 independence), + indicates establishment of continuously proliferating cell line and − indicates cell line will not grow under specified conditions. To establish whether infected cells could produce tumors in nude mice, the number of mice that developed large, nonregressing tumors at the site of subcutaneous injection of cells was shown. To determine whether conditioned medium could support Ba/F3 cell proliferation, conditioned medium was harvested from helper-virus-free cell lines at high density and passed through a 0.45-μm filter.
the pathogenesis of CML. Given that P210 will not transform fibroblasts, the requirement for transformation of adherent and nonadherent cell types by abl proteins appear to be distinct.

chromosome with virtually every case of CML and the similarity of P210

FIG. 3. Proliferation assay of Ba/F3 cell lines. Effect of dilution of conditioned medium on proliferation of various Ba/F3 cell lines. Conditioned medium is from the WEHI-3B cell line, a source of IL-3, unless otherwise noted. (A) •, Uninfected cells; ○, pWE-infected cells; ■, pWEgab-infected cells; □, pWE210-infected cells; ○, uninfected Ba/F3 cells plus conditioned medium from pWE210-infected Ba/F3 cells; □, uninfected Ba/F3 cells plus conditioned medium from pWEgab-infected Ba/F3 cells; ○, uninfected cells assayed in presence of 10 μg of control IgG (anti-epidermal growth factor receptor antibody); □, uninfected cells assayed in 3% (vol/vol) WEHI-3B conditioned medium to dilution of a neutralizing anti-IL-3 IgG; ■, pWEgab-infected cells assayed in medium lacking IL-3 to the indicated dilution of neutralizing anti-IL-3 IgG; ○, pWE210-infected cells assayed in medium lacking IL-3 to the indicated dilution of neutralizing anti-IL-3 IgG. Anti-IL-3 IgG (10 μg) neutralized 100% of the proliferative activity of 3 μl of WEHI-3B conditioned medium for Ba/F3 cells. Proliferation measurements have been normalized by subtracting the optical density (570 nm) for nonproliferating cells from each assay point and dividing by the optical density of maximally proliferating cells.

A previous study demonstrated the growth-promoting effects of P210 on early cells of the B-lymphoid lineage in the Whitlock-Witte bone marrow culture system (29). Not all of the clonal lines stimulated by P210 in that system were tumorigenic. Reminiscent of disease progression from chronic to acute phase, some clones progressed to tumorigenicty upon passage in culture, suggesting that secondary events were necessary in acquiring the full-tumorigenic phenotype. Whereas that study involved infection of primary bone marrow, the Ba/F3 cell line used in these experiments has been adapted for continuous growth in culture and may be more permissive for transformation.

This study demonstrates that the gene for P210ber/abl can function as a dominant oncogene. Like v-abl, it will transform the bone-marrow-derived IL-3-dependent Ba/F3 cell line to factor independence and tumorigenicity. P210 does not trigger the endogenous expression of IL-3 or other growth factors capable of stimulating Ba/F3 proliferation in an autocrine manner. The proliferation of the P210-transformed cells in the presence of a neutralizing anti-IL-3 antibody argues that the cells are not hyperresponsive to undetectable levels of IL-3 present in the growth medium. Rather, P210ber/abl must itself provide the stimulus for Ba/F3 cell proliferation normally provided through the IL-3 signal transduction pathway.

These experiments establish the oncogenic potential of P210ber/abl for the lymphoblastoid cell line Ba/F3. Preliminary results suggest that P210 will transform the IL-3-dependent mast cell line 32Dc13 to factor independence, thus demonstrating transformation for myeloid cell types (unpublished data). Unlike v-abl, P210 cannot transform NIH 3T3 fibroblasts unless it recombines with N-terminal gag sequences from the helper virus, which provides a myristoylation function critical for fibroblast transformation (14). Given that P210 will not transform fibroblasts, the requirement for transformation of adherent and nonadherent cell types by abl proteins appear to be distinct.
Fig. 5. Immunoprecipitation analysis of infected Ba/F3 cell lines. Cell extracts were incubated with anti-abl antisera (pEX4/5 mixture, ref. 23) and processed for in vitro immune-complex kinase reaction as described (22). Proteins were displayed by NaDodSO4/polyacrylamide gel electrophoresis and visualized by autoradiography. Each lane was normalized for total protein content. Lanes: 1, pWE210-infected cells maintained in IL-3 prior to selection for IL-3 independence; 2, pWE210-infected cells selected for IL-3-independent growth; 3, pWE210-infected cells maintained in IL-3 prior to selection for IL-3 independence; 4, pWE210-infected cells selected for IL-3-independent growth.

We thank David Schatz, Marjorie Oettinger, Stephen Smale, and Rick Van Etten for critical comments on the manuscript. This work was supported by a program project grant (CA38497) from the National Cancer Institute. G.Q.D. was supported by Public Health Service National Research Service Award 2T 32 GM07753-07 from the National Institute of General Medical Sciences.