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 P160bc/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 interleukin 3-dependent Ba/F3 cell line. As for P160bc/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 arose by substitution of the c-abl exon 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. Guild and R. C. Mulligan, Whitehead Institute) by using BamHI link-

ers. The resulting size for the pWEgab construct was 8.7 kb from the 5' long terminal repeat (LTR) to the 3' LTR. The full-length P210bcr/abl cDNA 172/215 (17) was cloned into pWE by using Bcl I linkers. The resulting LTR–LTR size for the pWE210 construct was 11 kb. Viral stocks were generated by the following methods. Replication-competent retroviral complexes were made by cotransfection of the constructs with helper Moloney murine leukemia virus DNA (p24AP, ref 18) onto NIH 3T3 cells. Alternatively, helper-free retroviral stocks were made by transfecting the constructs onto the packaging line ψ-2 (19) and infecting tunicamycin-treated ψ-2 cells with a transient viral harvest from the transfected ψ-2 cells. Retroviral supernatants from G418-resistant producer populations were harvested after 18 hr and passed through a 0.45-μm filter. Viral supernatants were titered for G418 resistance by infection of NIH 3T3 fibroblasts. All retroviral producer cell lines had comparable titers in the range of 105 G418-resistant colony-forming units/ml. Ba/F3 is a murine bone marrow-derived cell line dependent on IL-3 for viability and proliferation (15). Ba/F3 cells are classified as early cells of the lymphoblastoid lineage by virtue of their low-level expression of the B-cell-specific B220 antigen (unpublished data) and the germ-line configuration of their immunoglobulin loci. Ba/F3 cells were maintained in standard RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 10% (vol/vol) conditioned medium from the WEHI-3B cell line (as a source of IL-3, ref. 20). Ba/F3 cells are able to grow in medium supplemented with fetal calf serum and purified recombinant IL-3 alone (unpublished data). Ba/F3 cells not only fail to proliferate but also rapidly die in the absence of an exogenous source of IL-3. Numerous experiments with the Ba/F3 cell line conducted in this laboratory have failed to generate any spontaneous IL-3-independent clones. Ba/F3 cells (2 × 106 cells) were infected by incubation with 2 ml of viral supernatant and Polybrene at 8 μg/ml for 2 hr or by a 48-hr cocultivation with helper-free packaging lines in the presence of Polybrene at 2 μg/ml. Infected Ba/F3 cells were maintained in the presence of WEHI-3B conditioned medium for 48 hr and then selected for retroviral infection in G418 at 2 mg/ml. G418-resistant populations were washed twice with isotonic phosphate-buffered saline (PBS) before plating in medium lacking a source of IL-3 to select for IL-3-independent growth.

Assay of Cell Proliferation. Cell proliferation was assessed by an adaptation of the tetrazolium dye reduction assay of Mosmann (21). Relevant Ba/F3 cell lines were washed twice with PBS and resuspended at 5 × 105 cells per ml in RPMI medium containing 5% (vol/vol) fetal calf serum. Cells were

Abbreviations: CML, chronic myelogenous leukemia; A-MuLV, Abelson murine leukemia virus; IL-3, interleukin 3; LTR, long terminal repeat.

*In this report, lowercase italic gene symbols will be used for all genes, regardless of species of origin.
dispersed 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 × 10⁵ cells were injected subcutaneously into young (<12 week old) nude mice (BALB/c-μNcr-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 P121[gag/v-abl] or the pWEgab virus (encoding P160[NEO/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 P210[gag/v-abl] or P160[NEO/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.
IL-3-independent cell lines passed the G418-resistance marker upon infection of NIH 3T3 cells, but only virus rescued from pWEgab-infected cell lines was able to transform NIH 3T3 cells (Table 1). The properties of the rescued virus matched those of the original viral stock used to effect the IL-3-independent phenotype for Ba/F3 cells.

Cell extracts of relevant cell lines were immunoprecipitated with anti-abl antisera (23) and processed for the immune-complex kinase reaction with [$\gamma$-32P]ATP. The precipitated proteins were displayed by NaDodSO4/polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 5). Cell lines infected with the pWE210 and pWEgab viruses and selected in G418 expressed the P210(bcr/abl) and P160(ng/ab) proteins, respectively. IL-3-independent cell populations invariably expressed higher levels of abl protein, suggesting that IL-3 independence requires high-level expression of abl protein.

IL-3-independent cell lines expressing P210(bcr/abl) and P160(ng/ab) (either with helper virus or helper-free) were assayed for their tumorigenicity in nude mice. The parental Ba/F3 cells, or Ba/F3 cells infected with the pWE virus alone, did not form tumors upon subcutaneous challenge. The IL-3-independent, pWE210-infected, and pWEgab-infected cells readily formed tumors at the site of subcutaneous injection that were of short latency, nonregressing, and ultimately lethal to the host (Table 1).

**DISCUSSION**

A common denominator in CML is the Philadelphia chromosome, a t(9;22) that is detectable in a high proportion (>90%) of patients (24). The Philadelphia translocation juxtaposes c-abl protooncogene sequences on chromosome 9 with a locus on chromosome 22 (bcr) that encodes a protein of unknown function (25). The ability to detect the molecular rearrangement of bcr and abl in a proportion of the CML patients for whom cytogenetic evidence of the Philadelphia chromosome is lacking (26) further strengthens the association of the aberrant chromosome with the disease. The product of the Philadelphia chromosome, the P210(bcr/abl) fusion protein, resembles the P160(ng/ab) oncogene product in enzymatic and structural properties. The c-abl sequences that are deleted in the formation of P210 and P160 v-abl share homology with nonreceptor tyrosine kinases (e.g., src and fps), phospholipase C, and the avian v-crk oncogene (27, 28). Simple deletion of this region within the c-abl type IV sequence is sufficient to activate the transforming potential of c-abl for fibroblasts and lymphoid cells (P. Jackson and D.B., unpublished results). The association of the Philadelphia

**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 Secondary, - IL-3/ + G418</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>-</td>
<td>0/8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWE</td>
<td>-</td>
<td>0/7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWEgab</td>
<td>P160(ng/ab)</td>
<td>0/9.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWE210</td>
<td>P210(bcr/abl)</td>
<td>-</td>
<td></td>
<td></td>
<td></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 over the number of mice challenged 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. - Cells could not proliferate. To establish properties of rescued virus, Ba/F3 cell lines were superinfected with helper Moloney murine leukemia virus. Rescued retroviral supernatant was passed through a 0.45-µm filter before infection of NIH 3T3 cells. Infected cells were assayed for passage of the G418-resistance (Neo) gene by selection in G418 at 1 mg/ml or were allowed to grow to confluence and examined for the appearance of foci of transformed cells. +, Virus has indicated property; -, virus lacks the property.
chromosome with virtually every case of CML and the similarity of P210\textsuperscript{bcr/abl} to the v-\textit{abl} oncogene product of the Abelson murine leukemia virus strongly implicates P210 in the pathogenesis of CML.

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 tumorigenicity 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 P210\textsuperscript{bcr/abl} can function as a dominant oncogene. Like v-\textit{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, P210\textsuperscript{bcr/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 P210\textsuperscript{bcr/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-\textit{abl}, P210 cannot transform NIH 3T3 fibroblasts unless it recombines with N-terminal gag sequences from the helper virus, which provides a myristylation function critical for fibroblast transformation (14). Given that P210 will not transform fibroblasts, the requirements 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 antiserum (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, pWEgab-infected cells selected for IL-3 independence; 4, pWEgab-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.