c-RAF-1 Serine/Threonine Kinase Is Required in BCR/ABL-dependent and Normal Hematopoiesis

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Abstract

BCR/ABL oncogenic tyrosine kinase is responsible for initiating and maintaining the leukemic phenotype of Philadelphia chromosome-positive cells. c-RAF-1 serine/threonine kinase is known to be activated by receptor and nonreceptor tyrosine kinases. To determine whether c-RAF-1 plays a role in the growth of BCR/ABL-dependent cells, we examined whether c-RAF-1 associates with and/or is regulated by BCR/ABL and, if so, whether this interaction is functionally significant for BCR/ABL-dependent growth of chronic myelogenous leukemia cells and for growth factor-dependent proliferation of normal bone marrow cells. We show that c-RAF-1 enzymatic activity is regulated by BCR/ABL, although the protein does not associate with BCR/ABL. Downregulation of c-RAF-1 expression with antisense oligodeoxynucleotides or cDNA constructs, and inhibition of c-RAF-1 activity by its dominant negative mutants, inhibited both BCR/ABL-dependent growth of chronic myelogenous leukemia cells and growth factor-dependent proliferation of normal hematopoietic progenitors and the MO7 cell line without affecting the BCR/ABL- and growth factor-independent proliferation of HL-60 cells. These results indicate that c-RAF-1 plays an important role in Philadelphia chromosome-positive and normal hematopoiesis.

Introduction

BCR/ABL oncogenic tyrosine kinases, formed as a consequence of the 9;22 chromosomal translocation, cause CML1 or acute lymphatic leukemia (1, 2) and are necessary for the growth of Ph1-positive malignant cells (3). Cytoplasmic BCR/ABL (4) activates signaling pathways transducing oncogenic signals from the cytoplasm to the nucleus. We have demonstrated previously that BCR/ABL maintains RAS in the active form, a requirement for the proliferation of Ph1-positive cells (5). Proliferation of normal hematopoietic cells in the presence of growth factors also depends on RAS (6). The direct RAS downstream effector, p74rel1 serine/threonine protein kinase (c-RAF-1), is encoded by the c-raf-1 proto-oncogene (7). c-RAF-1 binds to activated (GTP-bound) RAS and to MAPK kinase (8), resulting in the activation of the latter and, in turn, of the MAPKs (9). Although it has been suggested that MAPKs regulate growth factor-dependent proliferation of normal hematopoietic cells (10), their role in BCR/ABL-dependent growth is questionable (11). Because RAS-dependent activation of MAPK could be c-RAF-1-dependent or independent (12), we analyzed the role of c-RAF-1 in the leukemic BCR/ABL-dependent and normal growth factor-dependent hematopoiesis.

Materials and Methods

Cells. Marrow cells were obtained by aspiration from the iliac crest of healthy individuals and CML patients after informed consent. Light density mononuclear cells were separated on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient. Those from healthy individuals were enriched for hematopoietic progenitors after removing adherent cells and T lymphocytes (A" T"NBMC as described (6)). BV173 (CML-BLC) and HL-60 (acute promyelocytic leukemia) cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co.), L-glutamine (GIBCO-BRL, Grand Island, NY), and penicillin/streptomycin (GIBCO-BRL) (RPMI complete medium). The MO7 growth factor-dependent myeloid cell line was maintained in Iscove modified Dulbecco’s medium (IMDM) complete medium supplemented with 10 units/ml of rhuIL-3 (Genetics Institute, Cambridge, MA). The parental DAGM murine growth factor-dependent cell line and DAGMCR/ABE, a BCR/ABL-dependent cell subline (kind gift of Dr. C. Sawyers, University of California-Los Angeles, Los Angeles, CA), were maintained in RPMI complete medium with or without 15% WEHI-CM added as a source of growth factors.

ODNs. ODNs were synthesized on a DNA synthesizer (Model 308B, Applied Biosystems, Inc., Foster City, CA) by means of β-cyanoethyl-phosphoramidite chemistry. The sequences of 18-mer c-raf-1-specific sense and antisense ODNs in 5’ to 3’ orientation are ATGTGACCGCTTCCCGCT and AGCGGGAGGCGGTCACAT, respectively (nucleotides 5-23 of published c-raf-1 cDNA) (7). The sequence of 5’ and 3’ primers and the probe of Neo-R-encoding gene are: CTCGTCAGAAAGGCGATAGA (nucleotides 351-371); TCCTTTGGGAAAAATGGCCGG (nucleotides 530-550); and GGTTAGCAGACCTGATGCTC (nucleotides 461-481), respectively, according to the GenBank sequence (accession no. VB0064).

Antisense and Sense Constructs. The 302-nt fragment of human c-raf-1 cDNA (nucleotides 141-442) was cloned into the pcDNA3 plasmid in the EcoRV site in sense and antisense orientation (pcDNA3-RAF/S or pcDNA3-RAF, respectively).

DNMs. Full-length WT human c-raf-1 cDNA was obtained from Dr. G. Cooper (Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA). The cDNAs of human c-raf-1 K375W and S621A kinase-inactive DNMs were kindly provided by Dr. D. Morrison (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). The filled Ndel-XbaI fragments (encoding amino acids 1-648) of WT and DNMs used for transfections were cloned under the CMV promoter into pcDNA3 plasmid in the BamHI site.

DNMs Treatment of the Cells. Cells were incubated with ODNs as described previously (13). Briefly, 105 primary cells and 106 cell line cells in 0.4 ml complete medium were cultured in 24-well plates, with 80 μg/ml ODNs added on day 0 and 40 μg/ml added on days +1 and +2. rhuIL-3 or rhuEPO (2 units/ml; R&D Systems, Minneapolis, MN) were added as indicated. On day +3 the cells were plated in MethoCult H4230 (Stem Cell Technologies, Vancouver, BC) semisolid medium as described (6), or cell lysates were prepared (13) for analysis of c-RAF-1 expression.
cipitate (Imm) from BV173 cells; BCR/ABL was detected in total cell lysate and anti-ABL immunoprecipitate (Imm) from BV173 cells; BCR/ABL was detected on the same blot after stripping (lower panel).

**Transfections.** The indicated plasmids were transfected into the cells by electroporation as described previously (14). After 7-day selection in RPMI complete medium containing geneticin (G418, Calbiochem, San Diego, CA), cells were plated in MethoCult H4230 in the presence of G418, RNA was extracted, and cell lysates were prepared as described (6, 13). Expression of Neo-R mRNA and of c-RAF-1 protein was analyzed by RT-PCR followed by Southern blotting and SDS-PAGE followed by Western blotting, respectively. RT-PCR. RNA was extracted from 10^7 transfected cells collected after selection in G418-containing medium (15) and divided into two identical aliquots, which were analyzed for expression of Neo-R and β-actin (as an internal control) transcripts by RT-PCR, followed by Southern blotting with the γ-32P end-labeled probe, as described (6).

**Immunoprecipitation and Western Blotting.** p210BCR/ABL was immunoprecipitated from the BV173 cell lysate as described (13) with the use of anti-ABL mAb (gift of Dr. R. B. Arlinghaus, M. D. Anderson Cancer Center, Houston, TX). Cell lysates, after selection in G418 medium, were prepared from 2 × 10^6 cells as described (13), c-RAF-1 and heat shock protein 72/73 (HSP 72/73) or p210BCR/ABL as internal controls were detected in the cell lysates and in immunoprecipitates by SDS-PAGE, followed by Western blotting as described (13), with the use of polyclonal anti-RAF-1 serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal anti-HSP 72/73 (Oncogene Science, Inc., Uniondale, NY), and anti-ABL antibodies, respectively.

**Results and Discussion.**

Our previous findings that activated RAS is involved in the proliferation of Ph1-positive and normal hematopoietic progenitors (5, 6) led us to analyze the possible role of the p74raf-1 proto-oncogene (c-RAF-1), a direct downstream effector of RAS (8), in BCR/ABL-dependent and normal hematopoiesis. As shown in Fig. 1A, minimal, if any, c-RAF-1 kinase activity was detected in the anti-c-RAF-1 immunoprecipitates from the growth factor-starved DAGM parental cell line, whereas significant enzymatic activity was detected in precipitates from the p210BCR/ABL-expressing cell subline. However, no c-RAF-1 was detected associated with p210BCR/ABL- expressing cell subline. No c-RAF-1 was detected with the use of Western immunoblotting in anti-ABL immunoprecipitates from BV173 cells (Fig. 1B). Together with previous reports of rapid c-RAF-1 phosphorylation and stimulation of its serine/threonine protein kinase activity in response to growth factors (e.g., granulocyte-macrophage CSF, IL-3, stem cell growth factor, and EPO; Refs. 17, 18), the data indicate that c-RAF-1 is likely to be an important intermediate in transducing mitogenic signals in normal and Ph1-positive hematopoietic cells. To determine a direct role of c-RAF-1 in proliferation of these cells, we analyzed whether c-RAF-1 down-regulation had any effect on the proliferative ability of normal and CML cells. Antisense strategies (incubation with antisense ODNs or transfection with an antisense construct) or functional inhibition of its activity with kinase-inactive DNMs were used for this purpose. In cells incubated with c-raf-1 antisense ODNs or transfected with a c-raf-1 antisense cDNA, expression of c-RAF-1 protein was down-regulated as compared to controls. As an example, only the data from HL-60 cells are shown (Fig. 2, A and B, left). Colony formation by CML primary cells and the Ph1-positive BV173 cell line, which depends on BCR/ABL for proliferation (3, 19), was inhibited in the same conditions, whereas that by...
the BCR/ABL- and growth factor-independent HL-60 cells was not affected (Table 1). Levels of Neo-R mRNA transcripts were similar in all conditions (Fig. 2C, left), excluding that the observed differences in clonogenic efficiency were due to quantitatively different expression of this gene. The observation that HL-60 cell proliferation is c-RAF-1 independent, together with our previous data indicating that these cells are also independent of RAS (5), a c-RAF-1 direct regulator, suggests that RAS/c-RAF-1-independent mechanism(s) such as c-myc amplification (20) are involved in the regulation of HL-60 cell proliferation. Colony formation from A-T NBMC and the MO7 growth factor-dependent cell line in the presence of rhuh-3 and rhuEPO was also abrogated upon c-raf-1 antisense ODN treatment (Table 1). Antisense ODNs also inhibited colony formation by stem cell growth factor-treated MO7 cells and by granulocyte-macrophage CSF or macrophage-CSF-treated A-T NBMC (not shown). These results demonstrate that c-RAF-1 expression is necessary for proliferation of both Ph-positive and of normal hematopoietic cells.

To define whether a functional c-RAF-1 serine/threonine protein kinase is required for the growth of Ph-positive and normal hematopoietic cells we used a dominant negative mutant strategy using two previously defined c-raf-1 DNMs. Transfection with kinase-inactive c-raf-1 DNMs K375W and S621A (21, 22) inhibited colony formation by primary CML-BC-2 patient cells by Ph1-positive cell line BV173, and by MO7 cells in the presence of IL-3, but not by HL-60 cells (Table 2). Expression of c-RAF-1 WT and DNMs proteins was analyzed in transfected cells after selection in G418. Expression of c-RAF-1 was increased significantly as detected by SDS-PAGE, followed by Western blotting with anti-c-RAF-1 antibody in cells transfected with WT and DNMs compared to its expression in cells transfected with insertless plasmid (Fig. 2B, right). Similar levels of Neo-R mRNA transcripts determined by RT-PCR and Southern blotting (Fig. 2C, right) were present in each condition.

Taken together, our data indicate that functional c-RAF-1 serine/threonine protein kinase is required for the proliferation of normal growth factor-dependent and Ph1-positive BCR/ABL-dependent hematopoietic cells. Our data are in agreement with those of Kolch et al. (21), who demonstrated that c-RAF-1 protein kinase is required for mitotic maturation of Xenopus oocytes, using S621A DNAs.

RAS-stimulated NIH/3T3 cell growth using c-raf-1 antisense construct or the K375W DNM, and those of Fabian et al. (22), suggesting that c-RAF-1 is involved in mitotic maturation of Xenopus oocytes, using S621A DNM.

The mechanism of c-RAF-1 activation by BCR/ABL is unknown. Ligand-activated receptor tyrosine kinases or intracellular tyrosine kinases activate RAS (23, 24). Interaction of RAS with the N-terminal regulatory domain of c-RAF-1 results in its recruitment to the plasma membranes (25), activation by unknown RAS-independent mechanism, and stimulation of the downstream effector MAPK kinase (26). Both activated tyrosine kinases and activated RAS are necessary for activation of c-RAF-1 (16). BCR/ABL and ligand-activated hematopoietic growth factor receptors have tyrosine kinase activity or activate intracellular tyrosine kinases and stimulate RAS (23, 27), a process that may be responsible for c-RAF-1 activation in hematopoietic cells. The observation that both c-RAF-1 and BCR/ABL interact with a member of the 14-3-3 family of proteins (28, 29), which are essential for cell proliferation and determination of the timing of mitosis in yeasts (30), suggests that BCR/ABL may regulate c-RAF-1 activity via RAS and 14-3-3.

In summary, the data reported here provide direct evidence that the c-RAF-1 serine/threonine protein kinase plays a role in the growth of normal and Ph1-positive hematopoietic cells. Additional studies are necessary to dissect the molecular mechanism(s) that regulate c-RAF-1 activity in BCR/ABL-dependent leukemic cells and growth factor-dependent normal hematopoietic cells.

### Acknowledgments

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### References


### Table 2 Effect of inhibition of c-RAF-1 activity on proliferation of BCR/ABL- or growth factor-dependent hematopoietic cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of colonies</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML-BC-2</td>
<td>253 ± 55</td>
<td>72/81 (P &lt; 0.001)</td>
</tr>
<tr>
<td>BV173</td>
<td>1198 ± 187</td>
<td>96/95 (P &lt; 0.001)</td>
</tr>
<tr>
<td>MO7</td>
<td>78 ± 13</td>
<td>85/92 (P &lt; 0.015)</td>
</tr>
<tr>
<td>HL-60</td>
<td>1308 ± 149</td>
<td>128/138 (NS)</td>
</tr>
<tr>
<td>ODNs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CML-CP</td>
<td>35 ± 10</td>
<td>62 (P &lt; 0.001)</td>
</tr>
<tr>
<td>CML-AP</td>
<td>95 ± 14</td>
<td>64 (P &lt; 0.001)</td>
</tr>
<tr>
<td>CML-BC-1</td>
<td>102 ± 22</td>
<td>79 (P &lt; 0.001)</td>
</tr>
<tr>
<td>CML-BC-2</td>
<td>425 ± 45</td>
<td>82 (P &lt; 0.001)</td>
</tr>
<tr>
<td>A-T NBMC IL-3</td>
<td>202 ± 18</td>
<td>53 (P &lt; 0.001)</td>
</tr>
<tr>
<td>A-T NBMC EPO</td>
<td>305 ± 37</td>
<td>56 (P &lt; 0.001)</td>
</tr>
<tr>
<td>BV173</td>
<td>2973 ± 316</td>
<td>92 (P &lt; 0.001)</td>
</tr>
<tr>
<td>MO7</td>
<td>359 ± 137</td>
<td>58 (P &lt; 0.001)</td>
</tr>
<tr>
<td>HL-60</td>
<td>1332 ± 258</td>
<td>6 (NS)</td>
</tr>
</tbody>
</table>

### Notes

* The indicated cell types were transfected with c-raf-1 antisense (AS) or sense (S) ODNs, or were transfected with insertless pDNA3 plasmid (control) or plasmid containing a 302-bp c-raf-1 cDNA segment in the sense or antisense orientation. Cells (105; CML-BC or A-T NBMC; 106; BV173, HL-60, or MO7) were plated in MethoCult H4230 (ODNs group) or selected in G418 and then plated (construct group). The number of colonies was scored after 9-12 day culture. Values are mean ± SD, duplicate cultures from 4 different experiments.

* Percentage of inhibition of colony formation by antisense ODNs or antisense construct in comparison with sense ODNs or sense construct, respectively. Numbers in parentheses, statistical significance (t test); NS, not significant.
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