

# Negative Regulation of p120GAP GTPase Promoting Activity by p210<sup>bcr/abl</sup>: Implication for RAS-dependent Philadelphia Chromosome Positive Cell Growth

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## Summary

The p210<sup>bcr/abl</sup> tyrosine kinase appears to be responsible for initiating and maintaining the leukemic phenotype in chronic myelogenous leukemia (CML) patients. p21<sup>ras</sup>-p120GAP interactions play a central role in transducing mitogenic signals. Therefore, we investigated whether p21<sup>ras</sup> and p120GAP are regulated by p210<sup>bcr/abl</sup>, and whether this activation is functionally significant for CML cell proliferation. We report that transient expression of p210<sup>bcr/abl</sup> in fibroblast-like cells induces simultaneous activation of p21<sup>ras</sup> and inhibition of GTPase-promoting activity of p120GAP, and confirm these data showing that downregulation of p210<sup>bcr/abl</sup> expression in CML cells with *bcr/abl* antisense oligodeoxynucleotides induces both inhibition of p21<sup>ras</sup> activation and stimulation of GTPase-promoting activity of p120GAP. Tyrosine phosphorylation of two p120GAP-associated proteins, p190 and p62, which may affect p120GAP activity, also depends on p210<sup>bcr/abl</sup> tyrosine kinase expression. Direct dependence of these effects on the kinase activity is proven in experiments in which expression of c-MYB protein in fibroblast-like cells or downregulation of c-MYB expression resulting in analogous inhibition of CML cell proliferation does not result in the same changes. Use of specific antisense oligodeoxynucleotides to downregulate p21<sup>ras</sup> expression revealed a requirement for functional p21<sup>ras</sup> in the proliferation of Philadelphia chromosome-positive CML primary cells. Thus, the p210<sup>bcr/abl</sup>-dependent regulation of p120GAP activity is responsible, in part, for the maintenance of p21<sup>ras</sup> in the active GTP-bound form, a crucial requirement for CML cell proliferation.

The Philadelphia chromosome (Ph<sup>1</sup>) is present in most chronic myelogenous leukemias (CML) (1, 2) and in a cohort of acute lymphocytic leukemia patients (3). This translocation results in the juxtaposition of the *abl* gene, normally on chromosome 9, and the *bcr* gene on chromosome 22. The *bcr/abl* hybrid genes generate fusion proteins in which the NH<sub>2</sub>-terminal region of *c-abl* is replaced by *bcr* exon 3, exon 2 (p210<sup>bcr/abl</sup>), or exon 1 (p185<sup>bcr/abl</sup>). These fusion proteins can transform immature hematopoietic cells in vitro (4, 5), cause CML or acute leukemia-like syndromes in mice (6–9), and are necessary for the growth of human CML cells in vitro and in vivo (10, 11). The p210<sup>bcr/abl</sup> and p185<sup>bcr/abl</sup> proteins possess constitutive tyrosine kinase activity, which is necessary for the transforming potential of the chimeric gene (12) and appears to be activated by sequences in the first *bcr* exon (13). *bcr/abl* proteins are localized mainly in the cytoplasm

(14). The identification of the substrates involved in transducing the oncogenic signal initiated by the *bcr/abl* protein is likely to shed light on the mechanisms of leukemogenesis and may reveal new targets for antileukemia therapy.

The role of p21<sup>ras</sup> in signal transduction and cell growth is well established, based in part on the observations that functional wild-type activated p21<sup>ras</sup> (GTP-bound form) is required for mitogenic and oncogenic activity of tyrosine kinases (15, 16) and for the growth of normal human hematopoietic cells (17). p21<sup>ras</sup> activity is regulated by the opposing effects of guanine nucleotide exchange factors and GTPase activating proteins (GAPs). Exchange factors stimulate the exchange rate of GDP for GTP on p21<sup>ras</sup> and thereby activate it (18, 19). Activated receptor tyrosine kinases interact with and modulate the activity of the guanine nucleotide exchange factor Sos via the GRB-2 protein (20, 21). Recently, the physical interaction between p210<sup>bcr/abl</sup> and GRB-2, and its cooperation with p21<sup>ras</sup> to induce oncogenesis, have been demonstrated in primary murine bone marrow cell cultures and in rat fibroblasts (22).

<sup>1</sup> Abbreviations used in this paper: CML, chronic myelogenous leukemia; CML-BC, CML-blast crisis; CML-CP, CML-chronic phase; GAP, GTPase activating protein; Ph<sup>1</sup>, Philadelphia chromosome.

GAPs (p120GAP and neurofibromatosis type 1 [NF-1] protein) stimulate the slow intrinsic rate of GTP hydrolysis on p21<sup>ras</sup> and therefore act as negative regulators of p21<sup>ras</sup> function (23–26). p120GAP forms a complex with *bcr/abl* proteins through its Src homology (SH)2 domain (27). Tyrosine phosphorylation of p120GAP and/or of the two GAP-associated proteins p190 and p62 was described in cells expressing other activated cytoplasmic tyrosine kinases (29, 30) or after cytokine stimulation (31). Phosphorylation of p190 and p62 was found in all CML cases examined, whereas p120GAP phosphorylation occurred only in some cases. p120GAP complexed with p190 has lower GTPase-promoting activity than monomeric p120GAP (30), which may allow p21<sup>ras</sup> to remain in its active GTP-bound form. On the other hand, p120GAP is also a candidate as a p21<sup>ras</sup> effector protein and may function downstream of p21<sup>ras</sup> in the signal transduction pathway (32–34).

p120GAP may be involved in *bcr/abl*-dependent leukemogenesis, based on its physical interaction with the p210<sup>*bcr/abl*</sup> protein (27) and its requirement in Ph<sup>1</sup> cell growth (35), but it is also a well-known negative regulator of p21<sup>ras</sup>. Accordingly, we asked whether p21<sup>ras</sup> is activated by p210<sup>*bcr/abl*</sup> and is necessary for the proliferation of Ph<sup>1</sup> chromosome-positive, *bcr/abl*-dependent CML cells, and whether it regulates both p120GAP and p21<sup>ras</sup> to allow them to work in concert in *bcr/abl*-dependent leukemogenesis. We also investigated potential mechanisms of the *bcr/abl*-dependent p21<sup>ras</sup> activation, focusing on the two p120GAP-associated proteins, p190 and p62, which might be involved in the regulation of GTPase-promoting activity of p120GAP. To this aim, we transiently expressed p210<sup>*bcr/abl*</sup> in cells to examine the effects of this protein on the activation of p21<sup>ras</sup>, the GTPase-promoting activity of p120GAP, and the tyrosine phosphorylation of p190 and p62. To complement our findings using cells more relevant for the functional analysis of *bcr/abl*, similar experiments were also carried out in Ph<sup>1</sup> chromosome-positive CML cells in which p210<sup>*bcr/abl*</sup> expression was inhibited with *bcr/abl* antisense oligodeoxynucleotides. Finally, the role of p21<sup>ras</sup> in the proliferation of CML cells was directly assessed by down-regulating p21<sup>ras</sup> expression with antisense oligodeoxynucleotides. Our results confirm that the status of p21<sup>ras</sup> activation in CML cells depends on p210<sup>*bcr/abl*</sup> expression and produce the first direct demonstration that p210<sup>*bcr/abl*</sup> inhibits the GTPase-promoting activity of p120 GAP, thus maintaining p21<sup>ras</sup> in an active GTP-bound form in CML cells.

## Materials and Methods

**Cell Lines.** BV173 cells with a t(9;22) chromosomal translocation established from a patient with CML-blast crisis (CML-BC) (36), NB4 promyelocytic leukemia cells with a t(15;17) chromosomal translocation (37), HL-60 promyelocytic leukemia cells (38), and COS-7 simian fibroblast-like cells (39), were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Syrian hamster Tk-ts13 fibroblasts (40) were maintained in DMEM with 10% FBS.

**Primary Cells.** Mononuclear cells from CML-BC and CML-chronic phase (CP) patients (>50,000/ $\mu$ l white blood cells, >95%

Ph<sup>1</sup> metaphases) were obtained after separation of peripheral blood on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). Light-density mononuclear cells were incubated on petri dishes for 90 min at 37°C to remove most adherent cells. CD34<sup>+</sup> cells were isolated as described using hematopoietic cellular antigen 1 mAb and polystyrene magnetic beads (17). Recovery of CD34<sup>+</sup> cells from CML-BC and CML-CP varied between 8 and 41% and 6 and 20%, respectively.

**Antisense (AS) and Sense (S) Oligodeoxynucleotides.** The following phosphorothioate oligodeoxynucleotides, dissolved in IMDM were used: *bcr/abl*: 5'-AAGGGCTTCTTCCTTA-3' (b2/a2 AS) and 5'-TAAGGAAGAAGCCCTT-3' (b2/a2 S); *c-myb*: 5'-TATGCTGTGCCGGGGTCTTCGGGC-3' (AS) and 5'-GCCCCAAGACCCCGGCACAGCATA-3' (S); *H-ras*: 5'-CAGCTTATATTCGTCAT-3' (AS) and 5'-ATGACGGAATATAGCCTG-3' (S); *K-ras*: 5'-AAGTTTATATTCAGTCAT-3' (AS) and 5'-ATGACTGAATATAACTT-3' (S); and *N-ras*: 5'-CAGTTTGTACTCAGTCAT-3' (AS) and 5'-ATGACTGAGTACAACTG-3' (S). These compounds were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) by means of  $\beta$ -cyanoethylphosphorimidite chemistry. They have sequences complementary (or corresponding) to the breakpoint region of the *bcr/abl* transcripts (41), codons 2–9 of *c-myb* (42), and codons 1–6 of H-, K-, and N-ras mRNAs (43, 44, and 45, respectively).

**Transfections.** Cells were transfected using the calcium-phosphate precipitation method (46) with slight modifications. After transfection with either 10  $\mu$ g of pSV-*bcr/abl* plasmid containing the 7.0-kb *bcr* exon 3/*abl* exon 2 cDNA driven by SV-40 early promoter, pSV-*c-myb* plasmid containing a human *c-myb* full-length cDNA driven by the SV-40 early promoter (47), or the empty pSV plasmid, the cells were washed and incubated in serum-free medium supplemented with 0.1% BSA (Sigma Chemical Co.).

**Oligomer Treatment of Cells.** CML-CP or CML-BC cells (10<sup>5</sup>) were seeded into 24-well cell culture plates (Costar Corp., Cambridge, MA) in 0.4 ml IMDM supplemented with 2% heat-inactivated human AB serum and Hepes buffer. BV173, HL-60, and NB4 cells (10<sup>4</sup>/100  $\mu$ l/well) were placed in 96-well culture plates in RPMI medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. For protein studies, 5  $\times$  10<sup>6</sup> BV173 or HL-60 cells/20 ml medium were placed in 175-cm<sup>2</sup> LUX tissue culture flasks (Nunc, Inc., Naperville, IL). The first dose of sense or antisense oligodeoxynucleotides was added at the beginning of culture. The second and third doses (50% of the initial one) were added 24 and 48 h later. Control groups were left untreated. Where indicated, 50 U/ml IL-3 (Genetics Institute, Cambridge, MA) was added to the primary cell cultures together with the third dose of oligodeoxynucleotides. Primary cells were incubated for an additional 72 h before plating in 35-mm petri dishes. The cells in 96-well plates were counted on days 4, 6, and 8 and viability was assessed using the vital dye trypan blue. Cells in flasks were depleted of dead cells by density gradient centrifugation on Histopaque-1077, and counted before use.

**Colony Assays.** Duplicate cultures were prepared in semisolid methylcellulose medium (HCC 4230; Terry Fox Labs, Vancouver, Canada). IL-3 and human AB serum concentrations during culture were fivefold lower than those used during the oligodeoxynucleotide treatment. In some cultures, no IL-3 was added. Cultures were maintained for 10–12 d, after which plates were scanned with an inverted microscope and the number of colonies ( $\geq$ 50 cells) and clusters (8–40 cells) were determined.

**Immunoprecipitation and Western Blotting.** Cells (2  $\times$  10<sup>6</sup>) were solubilized in lysis buffer containing 10 mM Hepes, pH 7.5, 0.15 M NaCl, 10% glycerol, 10  $\mu$ g/ml each leupeptin and aprotinin

(Sigma Chemical Co.), 1 mM PMSF, 1 mM NaVO<sub>4</sub>, 1% NP-40, and 5 mM EDTA. Postnuclear lysates were, if indicated, immunoprecipitated (1 h, 4°C) with 5 μl of anti-human GAP rabbit serum (UBI, Inc., Lake Placid, NY) precoupled to protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ). The beads were washed twice with lysis buffer and twice with 10 mM Hepes, 0.15 M NaCl, and 0.2% NP-40. Immunoprecipitated proteins were eluted in sample buffer, separated on 7.5% SDS-PAGE, and transferred to nitrocellulose (Micron Separations Inc., Westboro, MA). Filters were saturated with 0.5% gelatin in Tris-buffered saline (TBS) and then incubated (12 h, 4°C) with affinity-purified antiphosphotyrosine rabbit polyclonal antibody (ICN Biomedicals, Inc., Costa Mesa, CA), anti-ABL antibody (48), anti-v-H-ras mAb (Oncogene Science Inc., Uniondale, NJ) or anti-c-MYB antibody (UBI), as indicated. Filters were washed five times with 0.25% Tween, 0.25% NP-40 in TBS buffer. Bound proteins were detected using the enhanced chemiluminescence Western blotting detection system (Amersham Corp., Arlington Heights, IL). When indicated, after stripping, the filters were blotted sequentially with anti-human heat-shock protein (HSP) 72/73 (Oncogene Science Inc.) or anti-human p120GAP antiserum, as controls for loaded protein amounts.

**GTP-bound p21<sup>ras</sup> Assay.** BV173 cells (10<sup>7</sup>) were labeled with [<sup>32</sup>P]orthophosphate (NEN Research Products, DuPont, Wilmington, DE) (3 h, 37°C) in phosphate-free medium, and then GTP/GDP-bound p21<sup>ras</sup> was determined as described (49). In transfected Tk-ts13 and COS-7 cells, GTP-bound p21<sup>ras</sup> was detected as described (50). Briefly, cells were washed with serum- and phosphate-free RPMI medium and incubated in 4 ml phosphate-free RPMI containing 0.25 mCi [<sup>32</sup>P]orthophosphate. After 3–4 h at 37°C, the cells were washed once with ice-cold PBS, and 0.5 ml ice-cold buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl<sub>2</sub>, 150 mM NaCl, 0.5% NP-40, 10 μg/ml aprotinin, 0.5 mM PMSF), and finally, 50 μg/ml anti-v-H-ras rat mAb was added. After 1 h at 4°C, cells were dislodged by trituration and transferred to microcentrifuge tubes. p21<sup>ras</sup> in the supernatant was immunoprecipitated using Protein A-agarose coupled to goat anti-rat IgG (Oncogene Science Inc.) and analyzed as described (49).

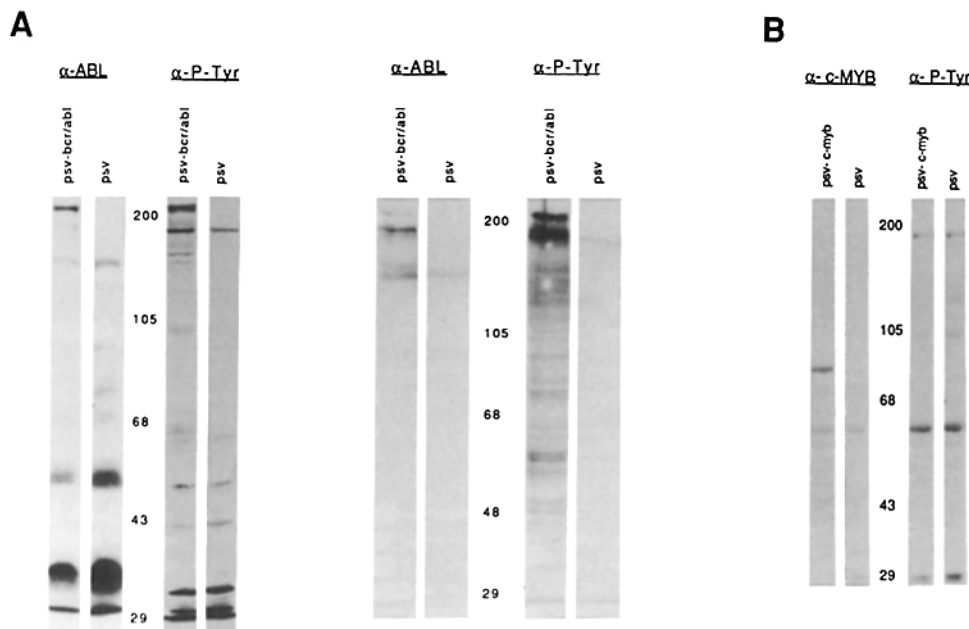
**Assay of GTPase-promoting Activity of p120GAP.** BV173 (10<sup>7</sup>),

Tk-ts13 (5 × 10<sup>6</sup>) and COS-7 cells (5 × 10<sup>6</sup>) were assayed for GTPase-promoting activity using purified p21<sup>N-ras</sup> protein (generous gift of Dr. F. McCormick, Onyx Pharmaceuticals, Richmond, CA) bound to α-[<sup>32</sup>P]GTP, as described (49), in the presence of 1 mM *n*-dodecyl-β-D-mannoside to inhibit NF-1 activity (51).

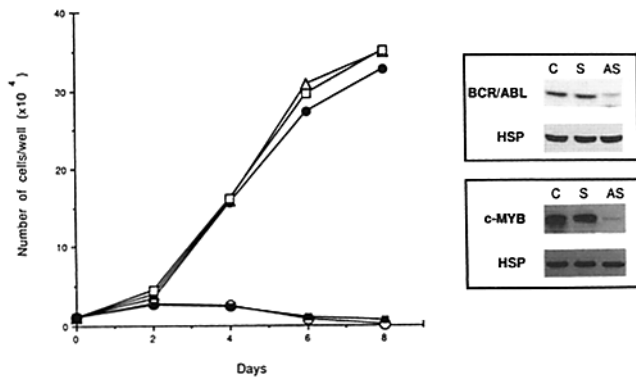
## Results

**p210<sup>bcr/abl</sup>-dependent Model Systems.** Downstream targets of p210<sup>bcr/abl</sup> tyrosine kinase were analyzed in Tk-ts13 and COS-7 cells transiently expressing a full-length *bcr/abl* cDNA driven by the early SV-40 promoter, and in the Ph<sup>1</sup>-positive cell line BV173 exposed to *bcr/abl*-specific phosphorothioate antisense oligodeoxynucleotides. Tk-ts13 and COS-7 cells were chosen for their high transfection efficiency and BV173 cells were selected for their exquisite sensitivity to p210<sup>bcr/abl</sup> deprivation. In both Tk-ts13 and COS-7 cells, p210<sup>bcr/abl</sup> expression levels, as detected by Western blotting and reaction with an anti-ABL mAb, were highest 36–48 h after transfection (Fig. 1 A), although the protein was already detectable 12 h after transfection (data not shown). The appearance of several tyrosine phosphorylated proteins (including one of 210 kD) in cells transiently expressing p210<sup>bcr/abl</sup> indicated that the protein had the expected kinase activity. In contrast, transfection of Tk-ts13 and COS-7 cells with the pSV vector only or with the pSV vector encoding c-MYB protein did not result in changes in protein tyrosine phosphorylation (Fig. 1 B).

Incubation of *bcr/abl*-dependent BV173 cells with *bcr/abl* antisense oligodeoxynucleotides resulted in a 80–90% decrease in expression of p210<sup>bcr/abl</sup>, but not of HSP 72/73 (Fig. 2), the p85 subunit of PI-3 kinase (11), p120GAP, or p21<sup>ras</sup> (data not shown). This treatment also led to complete inhibition of cell proliferation (Fig. 2). Proliferation of HL-60 and NB4 cell lines was not affected by treatment with *bcr/abl* antisense oligodeoxynucleotides (data not shown). BV173 cell proliferation was also inhibited after downregulation of c-MYB ex-



**Figure 1.** Expression of p210<sup>bcr/abl</sup> and c-MYB proteins and tyrosine phosphorylation of cellular proteins in cells transfected with *bcr/abl* cDNA or *c-myb* cDNA containing plasmids. (A) Tk-ts13 (left) and COS-7 cells (right) were transfected with pSV-*bcr/abl*, or pSV plasmids; (B) Tk-ts13 cells were transfected with pSV-*c-myb* or pSV plasmids. After 48 h, total cellular proteins were isolated from 10<sup>6</sup> cells, divided into two samples, and analyzed by Western blotting with anti-ABL (α-ABL), anti-c-MYB (α-c-MYB), or antiphosphotyrosine (α-P-Tyr) antibodies, as indicated.



**Figure 2.** Effect of *bcr/abl* and *c-myb* oligodeoxynucleotide treatment on BV173 cell proliferation and expression of p210<sup>bcr/abl</sup> or c-MYB proteins. BV173 cells were treated with *bcr/abl* sense (—□—), *c-myb* sense (—●—), *bcr/abl* antisense (—■—), or *c-myb* antisense (—○—) oligodeoxynucleotides (40+20+20 μg/ml). Control cells (—△—) were left untreated. The number of live cells was counted at the indicated days. Total cellular proteins were extracted from 10<sup>6</sup> cells after 72-h incubation with sense (S) or antisense (AS) oligodeoxynucleotides. Control (C) cells were left untreated. BCR/ABL and c-MYB proteins were detected in lysates of cells treated with *bcr/abl* or *c-myb* oligodeoxynucleotides, respectively, by Western blotting using specific antibodies to c-ABL or c-MYB. HSP 72/73 was detected on the same filters with anti-HSP 72/73 antibody. (Top) *bcr/abl* oligodeoxynucleotide-treated cells; (bottom) *c-myb* oligodeoxynucleotide-treated cells.

pression by antisense oligodeoxynucleotide treatment (Fig. 2). Thus, effects due to specific inhibition of p210<sup>bcr/abl</sup> tyrosine kinase expression can be distinguished from those that may be consequent to inhibition of cell growth per se.

**p21<sup>ras</sup> Activation in Cells after Induction or Inhibition of p210<sup>bcr/abl</sup> Expression.** Transient expression of p210<sup>bcr/abl</sup> tyrosine kinase resulted in a three- to fourfold increase in the amount of GTP-bound p21<sup>ras</sup> as measured in Tk-ts13 and COS-7 cells 48 h after transfection with pSV-*bcr/abl* plasmid, relative to the levels in cells transfected with the pSV empty plasmid (Table 1). The three- to fourfold increase in GTP-bound p21<sup>ras</sup> likely underestimates the effects of p210<sup>bcr/abl</sup>, because at most 70% of the cells are expected to be p210<sup>bcr/abl</sup> positive after transfection (40). Transient expression of c-MYB

**Table 1.** Activation of p21<sup>ras</sup> in Tk-ts13 and COS-7 Cells upon Expression of p210<sup>bcr/abl</sup> or c-MYB Proteins

Cells	pSV	pSV- <i>bcr/abl</i>	pSV- <i>c-myb</i>
COS-7	5.1 ± 0.8	23.7 ± 4.7*	ND
TK-ts13	7.1 ± 2.7	23.3 ± 1.0†	7.4 ± 0.6

The indicated cells were transfected with the pSV plasmid, empty or containing *bcr/abl* or *c-myb*, as indicated. p21<sup>ras</sup> activation was analyzed after 48 h.

The results are expressed as percent GTP-bound/GTP plus GDP-bound p21<sup>ras</sup>, mean ± SD, three experiments.

\*  $p = 0.03$ .

†  $p = 0.001$ , pSV-*bcr/abl* group compared with pSV group (Student's *t* unpaired test).

protein, which is essential for the growth of *bcr/abl*-dependent cells, but most likely acts downstream of p21<sup>ras</sup>, did not affect the ratio of GTP/GDP associated with p21<sup>ras</sup> (Table 1).

To further examine the role of p210<sup>bcr/abl</sup> in the activation of p21<sup>ras</sup> in leukemic cells carrying the Ph<sup>1</sup>-chromosome, the active GTP-bound p21<sup>ras</sup> was measured in BV173 cells exposed to *bcr/abl*-specific antisense oligodeoxynucleotides for 72 h to inhibit p210<sup>bcr/abl</sup> expression. After the culture, dead cells were depleted by density gradient centrifugation and biochemical analysis was conducted on an equal number of cells from treated and control cultures. Downregulation of p210<sup>bcr/abl</sup> expression was associated with a sevenfold decrease in GTP-bound p21<sup>ras</sup> as compared with untreated, or sense-treated cells (Table 2). In control experiments, downregulation of c-MYB expression was concurrent with inhibition of BV173 proliferation, but did not change the amount of GTP-bound p21<sup>ras</sup> (Table 2), thus demonstrating the specificity in the linkage between p210<sup>bcr/abl</sup> expression and p21<sup>ras</sup> activation.

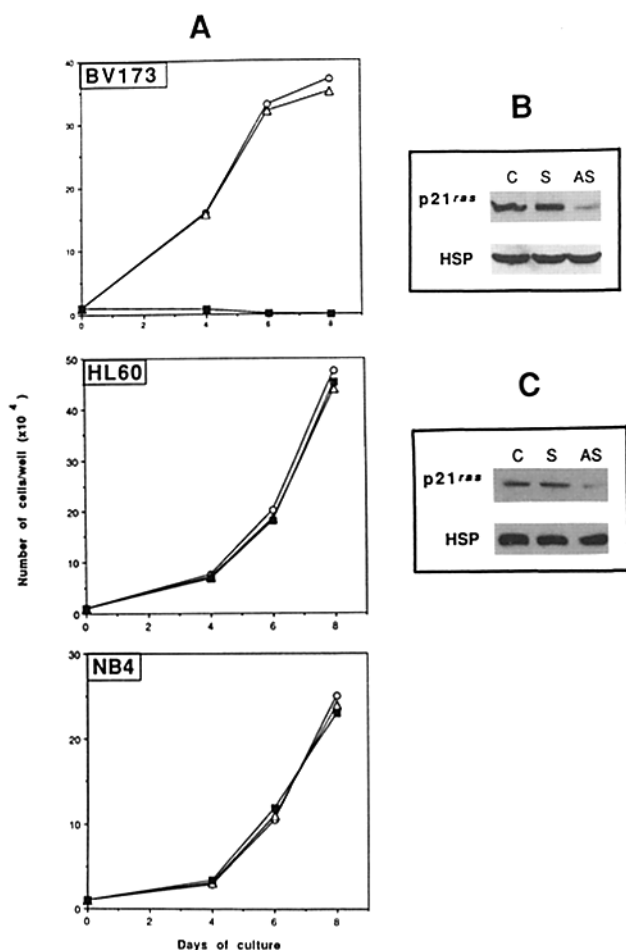
**Effect of Inhibition of p21<sup>ras</sup> Expression on Growth of *bcr/abl*-dependent Cells.** To obtain direct demonstration that p21<sup>ras</sup> plays a role in the growth of Ph<sup>1</sup> chromosome-positive cells, whose proliferation depends on p210<sup>bcr/abl</sup> expression, BV173 cells were treated with antisense oligodeoxynucleotides targeted against codons 1-6 of H-, K-, and N-*ras* exon 1. BV173 and primary CML cells expressed the mRNA of each of the three *ras* genes as detected by RT-PCR (data not shown). p21<sup>ras</sup> protein levels were decreased by treatment with the mixture of H-, K-, and N-*ras* antisense, but not with the mixture of sense oligodeoxynucleotides (Fig. 3 B). Proliferation of BV173 cells was concurrently completely inhibited by the antisense but not by the sense oligodeoxynucleotide mixture (Fig. 3 A). Of several different oligodeoxynucleotide concentrations tested, 8+4+4 μg/ml was the lowest that gave maximum inhibition of BV173 cells. In parallel experiments, the growth of two cell lines nondependent on *bcr/abl* or growth factors (HL-60 and NB4) was not affected (Fig. 3 A), even though p21<sup>ras</sup> expression was inhibited by the antisense oligodeoxynucleotide mixture in HL-60 cells (Fig. 3 C) and treatment within a fourfold higher concentration of

**Table 2.** Influence of p210<sup>bcr/abl</sup> or c-MYB Protein Downregulation on p21<sup>ras</sup> Activation in BV173 Cells

Oligomers	Oligodeoxynucleotide treatment		
	None	Sense	Antisense
<i>bcr/abl</i>	7.0 ± 0.8	7.4 ± 1.8	1.1 ± 1.5*
<i>c-myb</i>	7.9 ± 0.9	7.8 ± 1.5	7.6 ± 1.3

BV173 cells were exposed to the indicated oligodeoxynucleotides for 72 h, after which p21<sup>ras</sup> activation was determined. The results are expressed as percent GTP-bound/GTP plus GDP-bound p21<sup>ras</sup>, mean ± SD, three (*bcr/abl*) and two (*c-myb*) experiments, respectively.

\*  $p = 0.01$ , *bcr/abl* antisense versus *bcr/abl* sense and *c-myb* antisense group. (Student's *t* unpaired test).



**Figure 3.** Effect of p21<sup>ras</sup> downregulation on proliferation of *bcr/abl*-dependent and -independent cell lines. (A) The indicated cells were treated with a mixture of H-, K-, and N-*ras* sense (—○—) or antisense (—■—) oligodeoxynucleotides (40+20+20 μg/ml). Control (—Δ—) cells were left untreated. The number of live cells was scored at the indicated days (experiment representative of four performed). Total proteins (B and C) were isolated from 10<sup>6</sup> cells after 72-h incubation. p21<sup>ras</sup> protein and HSP 72/73 in BV173 (B) or HL-60 cells (C) were detected on the same filter by Western blotting using specific antibodies. Results are representative of two independent experiments.

oligodeoxynucleotides did not show any effect in these lines (data not shown).

To determine whether p21<sup>ras</sup> is also directly involved in the growth of Ph<sup>1</sup> chromosome-positive primary cells, CML-CP and CML-BC primary cells were incubated with the mixture of H-, K-, and N-*ras* antisense or sense oligodeoxynucleotides and their colony-forming ability was analyzed in a clonogenic assay with or without IL-3. Control cells were left untreated. Clonogenic growth of CML-CP and CML-BC cells was significantly inhibited by treatment with the antisense, but not sense oligodeoxynucleotide mixture (Table 3). All together, the results of these experiments indicate that proliferation of CML cells depends on p21<sup>ras</sup> expression.

#### GTPase-promoting Activity of p120GAP in Cells after Induc-

tion or Inhibition of p210<sup>bcr/abl</sup> Expression. GTPase-promoting activity of p120GAP was assessed in cell lysates from Tk-ts13 or COS-7 cells 48 h after transfection with pSV-*bcr/abl* plasmid or pSV empty plasmid. Assays were done, in the presence of 1 mM *n*-dodecyl-β-D-mannoside to inhibit NF-1 activity, using purified p21<sup>N-ras</sup> coupled to α-[<sup>32</sup>P]GTP as a substrate. p210<sup>bcr/abl</sup> expression and protein tyrosine phosphorylation were examined in each experiment. The amounts of α-[<sup>32</sup>P]GTP that remained bound to p21<sup>N-ras</sup> after a 10-min incubation with 20 and 75 μl of cell lysate from p210<sup>bcr/abl</sup>-expressing Tk-ts13 cells (60.1% and 45.1, respectively) were higher than those measured after incubation with lysates from cells not expressing p210<sup>bcr/abl</sup> (46.6 and 19.2%, respectively) (Fig. 4 A). Cell lysates from COS-7 cells expressing p210<sup>bcr/abl</sup> also showed reduction in GTPase-promoting activity of p120GAP (in 20 and 75 μl of cell lysate from p210<sup>bcr/abl</sup>-expressing cells, 67.2 and 64.8% of p21<sup>ras</sup> was in the GTP-bound form versus 62.4 and 51% of nonexpressing cells, respectively) (Fig. 4 B). These experiments suggest that transient expression of p210<sup>bcr/abl</sup> tyrosine kinase in transfected cells reduces the GTPase-promoting activity of p120GAP. After treatment with 20 and 75 μl of BV173 cell lysates obtained after 72-h incubation of the cells with *bcr/abl* phosphorothioate antisense oligodeoxynucleotides, only 44.0 and 6.9% of p21<sup>N-ras</sup> remained in the α-[<sup>32</sup>P]GTP-bound form. In contrast, 58 and 31.1% of p21<sup>N-ras</sup> was in α-[<sup>32</sup>P]GTP-bound form after incubation with 20 and 75 μl, respectively, of cell lysates obtained from sense oligodeoxynucleotide-treated cells (Fig. 4 C). Compared with sense oligodeoxynucleotide-treated cells, cell lysates from untreated cells had similar p120GAP activity. Downregulation of c-MYB expression was also associated with inhibition of BV173 cell growth, but this did not affect the GTPase-promoting activity of p120GAP, as compared with the control group (Fig. 4 C). This argues against the possibility that the observed changes in p120GAP activity were due to inhibition of cell proliferation, and confirms that downregulation of p210<sup>bcr/abl</sup> expression specifically results in enhancement of the GTPase-promoting activity of p120GAP.

**Phosphorylation of p120GAP and of two GAP-associated Proteins, p190 and p62 after Induction or Inhibition of p1210<sup>bcr/abl</sup> Expression.** Tyrosine phosphorylation of p120GAP and of two p120GAP-associated proteins, p190 and p62, was examined using antiphosphotyrosine antibody (α-P-Tyr) in Western blots of p120GAP immunoprecipitates from the cells transiently expressing p210<sup>bcr/abl</sup>. p120GAP was not phosphorylated in cells expressing p210<sup>bcr/abl</sup> tyrosine kinase, but tyrosine phosphorylation of p190 was three- to fourfold higher as compared with that in cells transfected with insertless plasmid (Fig. 5 A). No tyrosine phosphorylation of p62 was detected in cells transfected with the insertless plasmid even after long exposure of the blot, whereas the band corresponding to p62 was intense in cells transfected with the pSV-*bcr/abl* plasmid (Fig. 5 A). Equal amounts of immunoprecipitated p120GAP were detected on the same blot.

To analyze whether p210<sup>bcr/abl</sup> tyrosine kinase regulates tyrosine phosphorylation of p120GAP, p190, and p62 in CML cells, phosphorylation of these proteins was examined in BV173

**Table 3.** Effect of Inhibition of p21<sup>ras</sup> (H-ras, K-ras, and N-ras) Expression on In Vitro Colony Formation by CML Primary Cells

Cells	IL-3	Oligodeoxynucleotide treatment			Percent inhibition
		Control	Sense	Antisense	
CML-CP 1*	-	310 ± 16 <sup>†</sup>	317 ± 38	125 ± 35	61 ( <i>p</i> = 0.001) <sup>§</sup>
CML-CP 2	+	400 ± 26	458 ± 14	148 ± 23	68 ( <i>p</i> = 0.004)
CML-CP 3	+	120 ± 13	104 ± 4	29 ± 9	72 ( <i>p</i> < 0.001)
	+ <sup>  </sup>	27 ± 7	22 ± 4	4 ± 1	82 ( <i>p</i> = 0.001)
CML-CP 4	+	378 ± 21	378 ± 67	106 ± 4	72 ( <i>p</i> = 0.03)
CML-BC 1	-	225 ± 19	215 ± 24	92 ± 37	57 ( <i>p</i> = 0.001)
	+	1,120 ± 196	1,042 ± 189	454 ± 149	56 ( <i>p</i> < 0.001)
CML-BC 2	+	120 ± 17	119 ± 13	15 ± 7	87 ( <i>p</i> < 0.001)
CML-BC 3	+	507 ± 97	468 ± 101	113 ± 11	76 ( <i>p</i> < 0.001)
	+ <sup>  </sup>	67 ± 13	67 ± 13	31 ± 4	54 ( <i>p</i> < 0.001)
CML-BC 4	+	1,355 ± 426	1,280 ± 187	305 ± 13	76 ( <i>p</i> < 0.001)
	+ <sup>  </sup>	655 ± 35	630 ± 60	259 ± 16	59 ( <i>p</i> = 0.01)
CML-BC 5	+	597 ± 13	604 ± 54	155 ± 35	76 ( <i>p</i> < 0.001)
	+ <sup>  </sup>	240 ± 38	216 ± 18	58 ± 15	73 ( <i>p</i> < 0.001)
CML-BC 6	-	69 ± 9	59 ± 10	6 ± 1	90 ( <i>p</i> = 0.017)
	+	329 ± 72	331 ± 37	147 ± 27	56 ( <i>p</i> < 0.001)
	+ <sup>  </sup>	148 ± 29	138 ± 42	24 ± 11	83 ( <i>p</i> = 0.002)
CML-BC 7	+	359 ± 30	360 ± 45	105 ± 16	71 ( <i>p</i> = 0.017)
CML-BC 8	+	142 ± 8	107 ± 9	33 ± 3	69 ( <i>p</i> = 0.008)

160 or 80 µg/ml of the RAS oligodeoxynucleotides mixture was added at time 0 and the next two doses (each one half of the first dose) were added 24 and 48 h later. Cells ( $5 \times 10^4$  partially purified mononuclear cells or  $5 \times 10^3$  CD34<sup>+</sup> cells per plate) were plated after 120 h culture. *Bcr/abl* antisense oligodeoxynucleotide treatment inhibited colony formation by 40–50% (CML-CP) and 60–90% (CML-BC) in comparison with sense treatment.

\* PBMC were obtained from 12 CML patients, as indicated.

<sup>†</sup> Numbers are colonies per plate, mean of triplicates ± SD.

<sup>§</sup> Inhibition of colony formation by antisense versus sense oligodeoxynucleotide treatment. Statistical significance (by Student's *t* unpaired test) is reported in parentheses.

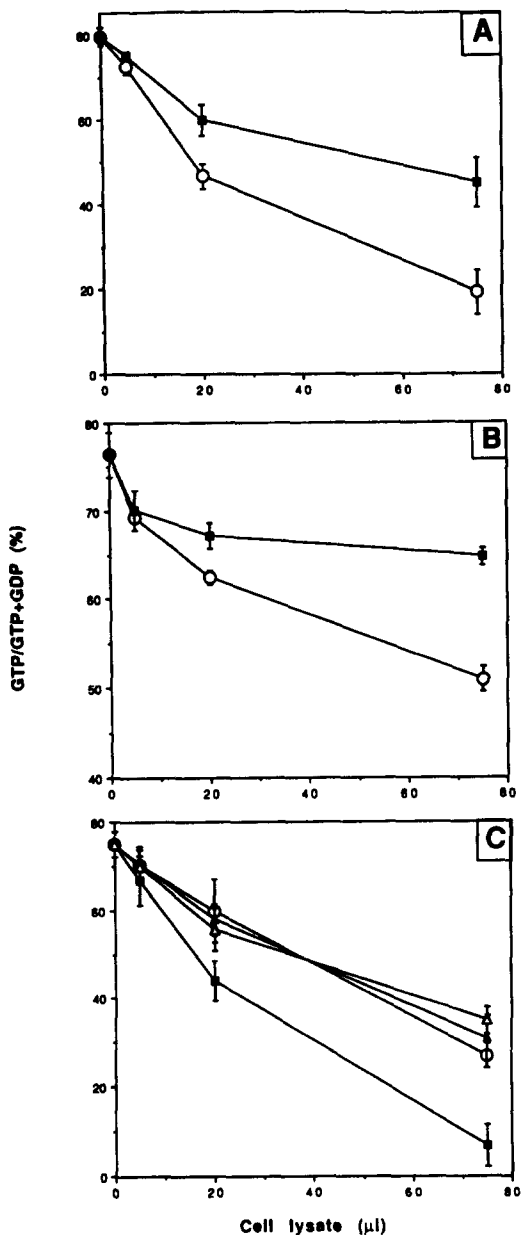
<sup>||</sup> CD34<sup>+</sup> cells were used.

cells in which p210<sup>bcr/abl</sup> expression was inhibited by incubation with *bcr/abl*-specific antisense oligodeoxynucleotides. The amount of tyrosine phosphorylated p190 and p62 immunoprecipitated with p120GAP in cells treated with *bcr/abl*-specific antisense oligomers was lower than that in cells treated with the sense oligomers (Fig. 5 B), although the amount of immunoprecipitated GAP was similar in each lane. Again, no tyrosine phosphorylation of p120GAP (Fig. 5 B) was detected in BV173 cells expressing p210<sup>bcr/abl</sup> tyrosine kinase (sense-treated cells). Similar results were obtained in two independent experiments with BV173 cells using antisense oligodeoxynucleotides at 80+40+40 or 40+20+20 µg/ml, and with primary CML-BC cells using oligodeoxynucleotide concentrations of 160+80+80 µg/ml (data not shown). To exclude the possibility that *bcr/abl* antisense oligomers inhibit phosphorylation of p190 and p62 nonspecifically, K562 cells (Ph<sup>1</sup> chromosome-positive with b3/a2 breakpoint (41)) were treated with b2/a2 antisense oligodeoxynucleotides which

do not interact with the b3/a2 transcripts and do not affect p210<sup>bcr/abl</sup> levels in K562 cells (Skorski, T., unpublished observation). Indeed, phosphorylation of p62 and p190 on tyrosine residues was not affected (data not shown). Moreover, inhibition of BV173 cell growth upon downregulated *c-MYB* expression by treatment with *c-myb* antisense oligodeoxynucleotides did not change the phosphorylation of p190 and p62 (Fig. 5 B). Thus, p210<sup>bcr/abl</sup> tyrosine kinase does not induce phosphorylation of p120GAP but regulates tyrosine phosphorylation of the p190 and p62 proteins, which are physically associated with p120GAP.

## Discussion

This study was undertaken to determine whether p21<sup>ras</sup> and p120GAP, two signal transduction pathway molecules possibly involved in *bcr/abl*-induced leukemogenesis, are regulated by p210<sup>bcr/abl</sup> tyrosine kinase. We used two separate



**Figure 4.** Effect of p210<sup>bcr/abl</sup> expression on GTPase-promoting activity of p120GAP. Tk-ts13 (A) and COS-7 cells (B) were transfected with pSV-*bcr/abl* (●) or pSV plasmids (○). BV173 cells (C) were treated with *bcr/abl* antisense (▲) *bcr/abl* antisense (■) or *c-myb* antisense (△) oligodeoxynucleotides (40+20+20 μg/ml), or left untreated (○). After 48 (A and B) or 72 h (C), cell lysates were prepared and assayed for GTPase-promoting activity on p21<sup>ras</sup> using recombinant p21<sup>Nras</sup> coupled to α-[<sup>32</sup>P]GTP. Results are expressed as percent GTP-bound/GTP plus GDP-bound forms of p21<sup>Nras</sup> from two independent experiments. Bars are SD.

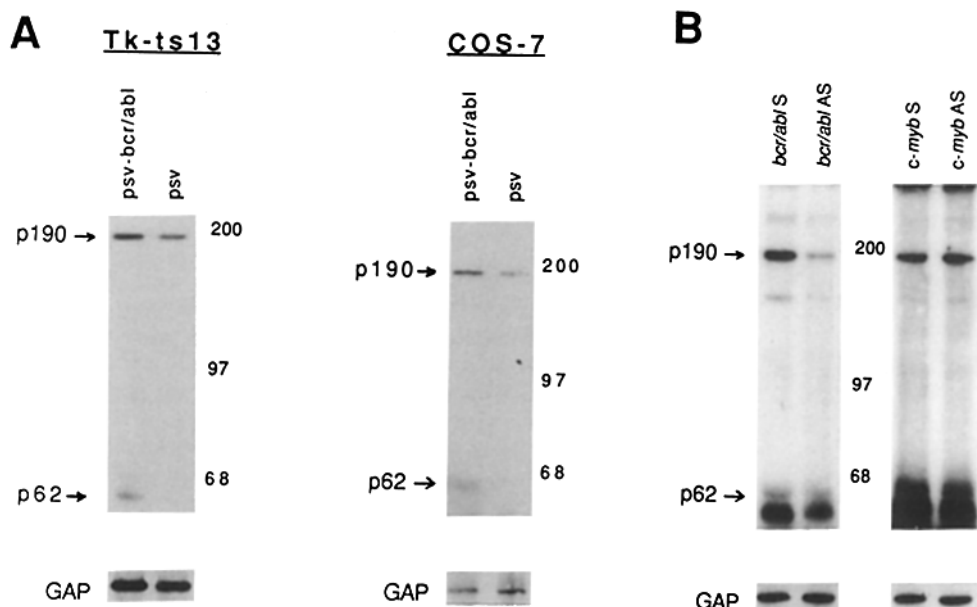
experimental approaches: transient expression of p210<sup>bcr/abl</sup> protein in fibroblasts and downregulation of p210<sup>bcr/abl</sup> expression in Philadelphia chromosome-positive cells by their exposure to antisense oligodeoxynucleotides directed against *bcr/abl* mRNA. Results obtained with both approaches indicated that the status of p21<sup>ras</sup> activation depended on p210<sup>bcr/abl</sup> expression. Transient expression of p210<sup>bcr/abl</sup> upon cDNA

transfection in Tk-ts13 and COS-7 cells stimulated p21<sup>ras</sup>, as indicated by the increased amount of its GTP-bound form. Conversely, selective inhibition of p210<sup>bcr/abl</sup> expression in CML cells by *bcr/abl*-targeted antisense oligodeoxynucleotides resulted in diminished GTP-bound p21<sup>ras</sup>. In control experiments, the human c-MYB protein, a transcriptional regulator that presumably functions only in the nucleus, did not affect p21<sup>ras</sup>. The antisense oligodeoxynucleotide strategy was previously employed by others (52, 53) to inhibit p21<sup>ras</sup> expression. Our results using this approach directly demonstrate that p21<sup>ras</sup> is necessary for the proliferation of CML cells and suggest that its activation by p210<sup>bcr/abl</sup> plays a crucial role in *bcr/abl*-induced leukemogenesis.

The possibility that an oncogenic point mutation in p21<sup>ras</sup>, rather than the stimulation of wild-type p21<sup>ras</sup> by p210<sup>bcr/abl</sup>, is responsible for its constitutive activation and consequent proliferation of CML cells, especially CML-BC, is unlikely because oncogenic point mutations in the *RAS* genes are rarely found in CML (54–56). N-*ras* point mutations have been detected in a very small group of CML-BC patients (56). We have detected no point mutations in N-*ras* exon 1 and exon 2 in several CML-BC patients whose N-*ras* we sequenced (Skorski, T., unpublished data). Thus, together with our previous findings that proliferation of growth factor-stimulated normal bone marrow progenitor cells depends on wild-type p21<sup>ras</sup> (17), the results reported here support a primary role for wild-type p21<sup>ras</sup> as a key signal transducing molecule in Ph<sup>1</sup> chromosome-positive hematopoietic cells. Presumably, in the leukemic cells, p21<sup>ras</sup> is abnormally activated by p210<sup>bcr/abl</sup>, resulting in disruption of the mechanisms regulating ligand-receptor-activated signal transduction pathways.

Tyrosine kinases might activate p21<sup>ras</sup> proteins through distinct pathways, depending on the cell type and on conditions that favor a particular pathway among several alternatives. p21<sup>ras</sup> activation correlates with decreased p120GAP activity in T and B cells after activation by PHA and antigen, respectively (49, 57). Binding of epidermal growth factor (EGF) to its receptor stimulates p21<sup>ras</sup> (58) by changing the intracellular localization of guanine nucleotide exchange factor, thus modulating its access to the substrate, and decreasing p120GAP GTPase-promoting activity (59). Nerve growth factor stimulates both exchange factor and GTPase-promoting activities (60), and p21<sup>ras</sup> activation is controlled by the balance in activity between these two regulatory proteins. The mechanism(s) of p21<sup>ras</sup> activation by p210<sup>bcr/abl</sup> in CML cells is poorly understood. However, a recent publication (22) suggests that the GRB-2/Sos pathway may be involved. The association of BCR/ABL protein with GRB-2 reported in that manuscript is required for the activation of the p21<sup>ras</sup> signaling pathway in transfected fibroblasts and for transformation of fibroblasts and murine bone marrow cultures.

Because p210<sup>bcr/abl</sup> is complexed with p120GAP in CML cells (28, 35) and activated p21<sup>ras</sup> (this report), we investigated whether the latter effect depends on modulation of GTPase-promoting activity of p120GAP. Using two distinct approaches to modulate p210<sup>bcr/abl</sup> expression i.e., transient expression in fibroblasts and inhibition by antisense oligode-



**Figure 5.** Effect of expression of p210<sup>bcr/abl</sup> and c-MYB on the phosphorylation of p190 and p62 p120GAP-associated proteins. (A) Tk-ts13 and COS-7 cells were transfected with pSV-bcr/abl or pSV empty plasmids; (B) BV173 cells were treated with 40, 20, and 20  $\mu$ g/ml of bcr/abl or c-myb antisense (AS) or sense (S) oligodeoxynucleotides, as indicated. p120GAP immunoprecipitates were obtained from cell lysates after 48-h (A) or 72-h (B) incubation and analyzed sequentially by Western blotting with antiphosphotyrosine (top) and anti-p120GAP antibodies (bottom).

oxynucleotides in CML cells, we demonstrate that p210<sup>bcr/abl</sup> negatively regulates the GTPase-promoting activity of p120GAP. Again, deregulation of c-MYB expression in CML cells did not affect p120GAP activity. These results strongly support the conclusion that activation of p21<sup>ras</sup> by p210<sup>bcr/abl</sup> tyrosine kinase in CML cells depends, at least in part, on inhibition of GTPase-promoting activity of p120GAP. It is possible that both p120GAP-dependent (this paper) and GRB-2/Sos-dependent (22) mechanisms of p21<sup>ras</sup> activation work in concert in bcr/abl-dependent leukemic cells. p210<sup>bcr/abl</sup> may activate p21<sup>ras</sup> via GRB-2/Sos and, at the same time, inhibit the rate of p21<sup>ras</sup> inactivation (hydrolysis of GTP-p21<sup>ras</sup> to GDP-p21<sup>ras</sup>), decreasing the GTPase-promoting activity of p120GAP. The existence of the GRB-2/Sos activation mechanism awaits, however, confirmation in Ph<sup>1</sup> chromosome-positive cells.

The mechanism(s) by which p210<sup>bcr/abl</sup> might regulate the GTPase-promoting activity of p120GAP was analyzed. Two proteins, p190 and p62, are tyrosine phosphorylated and are associated with p120GAP in cells expressing oncogenic tyrosine kinases, including p210<sup>bcr/abl</sup> (61, 30, 62). p190 was shown to inhibit in vitro GTPase-promoting activity when complexed with p120GAP (30). Our experiments expand this observation to show that p210<sup>bcr/abl</sup>, but not c-MYB, increases the proportion of tyrosine phosphorylated p190 and p62 detected in complex with p120GAP, which is not tyrosine phosphorylated. Elevated levels of tyrosine phosphorylated p190 complexed with p120GAP correlate with increased amount of GTP-bound p21<sup>ras</sup> (62, 63). This raises the possibility that p210<sup>bcr/abl</sup> inhibits the GTPase-promoting activity of p120GAP by increasing the tyrosine phosphorylation of p190 complexed with it. Alternatively, p210<sup>bcr/abl</sup> might bind p120GAP and inactivate it or make it less available for interaction with p21<sup>ras</sup>. The first mechanism was described for activated EGF receptor (59).

The consequence(s) of p21<sup>ras</sup>-p120GAP interaction is a matter of controversy (32, 64). The COOH-terminal region of p120GAP is sufficient to accelerate p21<sup>ras</sup> GTPase activity and therefore to inactivate p21<sup>ras</sup> (65). The NH<sub>2</sub>-terminal region contains two SH2 domains and an intervening SH3 domain, all implicated in the interactions between tyrosine kinases and their target molecules (66). p120GAP interacts with the domain of p21<sup>ras</sup> that has effector function (67), and it has been suggested that GAP serves both as a target for activated p21<sup>ras</sup> (33, 34) and as its negative regulator (25, 26). Hence, the transient interaction of p120GAP with p21<sup>ras</sup>-GTP at the plasma membrane might simultaneously induce GTP hydrolysis and generate a downstream signal involving a p120GAP-associated activity or protein. Indeed, p120GAP SH2-SH3 domains, or the SH3 domain per se, may transduce signals from p21<sup>ras</sup> to activate fos promoter in mammalian cells (33) and to block germinal vesicle breakdown induced by oncogenic H-ras in Xenopus oocytes (34). p21<sup>ras</sup>-GTP interacts with the COOH-terminal region of p120GAP and is consequently altered to p21<sup>ras</sup>-GDP. This reaction is thought to induce a conformational change(s) in the SH2-SH3 domains, allowing them to interact with phosphorylated proteins (33).

These observations may explain the role of p120GAP in p210<sup>bcr/abl</sup>-dependent cells both as signal transducer (35), and as negative regulator of RAS activity (this paper). Here, we report that the amount of tyrosine phosphorylated p190 and p62 complexed to p120GAP is increased by p210<sup>bcr/abl</sup>. This may allow the formation of p120GAP-p190 and/or p120GAP-p62 complexes which may either regulate the transduction of mitogenic signals through p21<sup>ras</sup> or transduce a signal from p21<sup>ras</sup> or directly from p210<sup>bcr/abl</sup>. p190 and p62 are localized both in the cytoplasm and in the nucleus (62, 68). p62 has extensive sequence homology with the putative hnRNP protein GRP33, binds to DNA and RNA (68), its



phosphorylation on tyrosine residues correlates with the transforming activity of p210<sup>bcr/abl</sup> (69), and it has been proposed to provide a link between phospholipase C (PLC $\gamma$ -1) and p21<sup>ras</sup> activation pathways (70). p190, in addition to regulating the GTPase-promoting activity of p120GAP, may function as a transcriptional repressor (61) and may serve as a bridge between *ras* and *rho* signal transduction pathways (71). Thus, it is possible that p62 and p190 have signal transducing functions on their own.

In summary, we directly proved the hypothesis that p21<sup>ras</sup>

expression is necessary for the *bcr/abl*-dependent proliferation of Ph<sup>1</sup> CML cells. Our data demonstrate that p210<sup>bcr/abl</sup> tyrosine kinase stimulates p21<sup>ras</sup> activity and inhibits GTPase-promoting activity of p120GAP, which may help p21<sup>ras</sup> to remain in an active GTP-bound form in CML cells. The amount of tyrosine phosphorylated p190 and p62 proteins detectable in complex with p120GAP is increased by p210<sup>bcr/abl</sup>, suggesting that these proteins may regulate GTPase-promoting activity of p120GAP and/or serve as signal transducing molecules in *bcr/abl*-dependent leukemogenesis.

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