p120 GAP Requirement in Normal and Malignant Human Hematopoiesis

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Summary

There is evidence to suggest that the p120 GAP (GAP), originally described as an inhibitor of p21*ras*, may also serve as a downstream effector of *ras*-regulated signal transduction. To determine whether GAP expression is required for the growth of human normal and leukemic hematopoietic cells, we used GAP antisense oligodeoxynucleotides to inhibit it and analyzed the effects of this inhibition on the colony-forming ability of nonadherent, T lymphocyte–depleted mononuclear cells and of highly purified progenitors (CD34+ MNC) obtained from the bone marrow and peripheral blood of healthy volunteers or chronic myeloid leukemia (CML, *bcr-abl*–positive) patients. The acute myelogenous leukemia cell line MO7, the Philadelphia[1] BV173 cell line, and the acute promyelocytic leukemia NB4 and HL-60 cell lines were similarly examined. GAP antisense treatment inhibited colony formation from normal myelo-, erythro-, and megakaryopoietic progenitor cells as well as from CML progenitor cells. Proliferation of MO7 (growth factor–dependent) and BV173 (*bcr-abl*–dependent) cells, but not that of NB4 and HL-60 (growth factor–independent) cells, was also inhibited, even though a specific downregulation of GAP was observed in each cell line, as analyzed by either or both mRNA and protein expression. Stimulation of MO7 cells with hematopoietic growth factors increased the expression of GAP as well as the levels of active GTP-bound p21*ras*. Stimulation of GAP expression was inhibited upon GAP antisense treatment. These data indicate that p120 GAP is involved in human normal and leukemic hemopoiesis and strongly suggest that GAP is not only a p21*ras* inhibitor (signal terminator), but also a positive signal transducer.

Proliferation and differentiation of hematopoietic cells are regulated by several growth factors that, upon interaction with specific cell surface receptors, activate signal transduction pathways in the cytoplasm and nucleus that affect the expression of proliferation- and/or differentiation-associated genes (1–4). Ligand-activated hematopoietic growth factor receptors possess intrinsic tyrosine kinase activity or associate with intracellular tyrosine kinases (4, 5), which in turn phosphorylate tyrosine residues of other cytoplasmic proteins (6, 7) in both normal and leukemic cells (8). The IL-2, IL-3, CSF-1, GM-CSF, epidermal growth factor (EGF)[1], stem cell factor (SCF), and platelet-derived growth factor (PDGF) receptors (9–13) and several oncogene products with constitutively enhanced tyrosine kinase activity (*fms*, *src*, *abl*, *bcr-abl*) (13–15) also activate p21*ras* proteins. These proteins are encoded by the H-, K-, and N-*ras* genes; they belong to a family of signal-transducing monomeric proteins with GTP-binding activity and appear to play a central role in signal transduction pathways (16). They bind guanine nucleotides with high affinity and hydrolyze GTP with low catalytic efficiency. In the GTP-bound form they serve as signal trans-

[1] *Abbreviations used in this paper:* AML, acute myelogenous leukemia; A-T-MNC, adherent cells and T lymphocytes; BC, blast crisis; CFU-Meg, CFU-megakaryocytic; CML, chronic myeloid leukemia; EGF, epidermal growth factor; Epo, erythropoietin; FBS, fetal bovine serum; GNRF, guanine-nucleotide releasing factor; HSP, heat shock protein; MNC, mononuclear cell; NF-1, neurofibromatosis type 1 protein; PDGF, platelet-derived growth factor; SCF, stem cell factor.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/12/1923/11 $2.00
Volume 178 December 1993 1923–1933

ducers (14, 17) but are inactive in the GDP-bound form. In mammalian cells, two proteins, p120 GAP and neurofibromatosis type 1 (NF-1) protein, inactivate $p21^{ras}$ (18) by inducing a 100-fold increase of the intrinsically low GTPase activity of $p21^{ras}$ which converts GTP-bound active $p21^{ras}$ to its inactive GDP-bound form by stimulation GTP-GDP exchange (17). Several lines of evidence suggest that, independently from its role as negative regulator, GAP, but not NF-1 protein (19), may also serve as a downstream target for $p21^{ras}$. Mutant forms of $p21^{ras}$ that lack effector function also fail to interact properly with GAP (20), suggesting that $p21^{ras}$ may regulate GAP. Oncogenic forms of $p21^{ras}$, whose GTPase activity is not upregulated by GAP, bind it with equal or greater affinity than wild-type $p21^{ras}$ (21). Cooperation between GAP and $p21^{ras}$ to inhibit coupling between muscarinic receptors and atrial potassium channels (22), in the RAS-dependent transactivation of a polyoma enhancer sequence (23), and in the activation of mitogen-activated protein (MAP) kinase (24), have also been reported. Finally, microinjection into oocytes of neutralizing anti-GAP Abs, or of Abs recognizing, and peptides corresponding to amino acid sequence of GAP SH3 domain, inhibits transforming v-H-*ras* p21-induced H1-kinase (25), and oncogenic H-ras–dependent germinal vesicle breakdown (26), respectively.

We have previously shown that $p21^{ras}$ plays an important role in the formation of normal and leukemic hematopoietic colonies (27, and our unpublished observations). To determine directly whether GAP is a signal transducer important for the growth of normal and/or malignant hematopoietic cells, we investigated colony formation and proliferation by these cells under conditions where GAP expression is inhibited. If GAP acts in hematopoietic cells primarily to inactivate $p21^{ras}$-GTP (activated by hematopoietic growth factors), then inhibition of GAP expression would be expected to increase the amount of GTP-bound $p21^{ras}$, to positively regulate signal transduction, and to increase, or not to affect, hematopoietic colony formation. If instead GAP is not only a signal terminator but also a signal transducer, inhibition of its expression may be expected to diminish the number of hematopoietic colonies stimulated by a hematopoietic growth factor(s), regardless of the persistence of $p21^{ras}$ in an active GTP-bound form.

Results of such analyses are reported in this paper.

Materials and Methods

Primary Cells. Marrows were obtained by aspiration from the iliac crest of healthy volunteers after informed consent. Light-density mononuclear cells (MNC), separated on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient, were enriched for hematopoietic progenitors after removing adherent cells and T lymphocytes (A-T-MNC) as described (28). A-T-MNC were used as such or were further enriched for immature hematopoietic progenitor ($CD34^+$) cells using HPCA-1 mAb (Becton Dickinson & Co., San Jose, CA) and Dynabead M-450 magnetic polystyrene beads coated with sheep anti-mouse IgG1 (Fc) (Dynal, Oslo, Norway). The yield of $CD34^+$ cells from A-T-MNC ranged between 0.5 and 3.2% of the starting MNC population. MNC from chronic myeloid leukemia (CML)-blast crisis (BC) patients were purified similarly. The yield of $CD34^+$ cells from primary cells of a CML-BC patient (UPN1) was 35.2%.

Cell Lines. MO7 acute myelogenous leukemia (AML) cells (29) were grown in IMDM supplemented with 10% fetal bovine serum (FBS) and IL-3, GM-SCF (Genetics Institute, Cambridge, MA), or SCF (Immunex, Seattle, WA). BV173 and K562 cells with a t(9;22) chromosomal translocation, established from patients with CML in lymphoid or myeloid BC, respectively (30, 31), NB4 promyelocytic leukemia cells with a t(15;17) chromosomal translocation (32), and HL-60 (33) promyelocytic leukemia cells, were maintained in RPMI 1640 supplemented with 10% FBS.

Oligodeoxynucleotides. These were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) by means of β-cyanoethyl-phosphorimidite chemistry. The sequences of GAP antisense and sense oligodeoxynucleotides are 5'-GGC CTC GGC CGC CAT CAT-3' and 5'-ATG ATG GCG GCC GAG GCC-3', respectively. They are complementary (or corresponding) to 18 nucleotides beginning with the initiation codon. GAP mRNA was detected using the RT-PCR technique with a 5' synthetic primer (5' TAA GAG AGA GTG ATC GGA GG 3') and a 3' synthetic primer (5' TGG CTC TGG TGG TGC AAC TG 3'). The amplification product was detected by hybridization to a synthetic oligomer included in the amplified segment (5' GGA GAT TAC TAC ATT GGT GGA AGA CGT TTT TCT TCA CTG 3') encompassing nucleotides 706–745 of the published sequence of human GAP cDNA (34). Computer search (FASTA) indicated that the GAP antisense oligomer sequence used is not complementary to any other known gene sequence. β-actin mRNA was amplified with primers derived from the published sequence of human β-actin and detected with a probe corresponding to nucleotides 258–296 (35).

Oligomer Treatment of the Cells. A-T-MNC (10^5), CML-BC (10^5), or $CD34^+$ (10^4) cells were seeded into 24-well culture plates (Costar Corp., Cambridge, MA) in 0.4 ml IMDM supplemented with 2% heat-inactivated human AB serum and Hepes buffer. Sense or antisense GAP oligodeoxynucleotides were added at a concentration of 160 μg/ml at the beginning of culture, and at 80 μg/ml 18 and 40 h later. Appropriate recombinant human growth factors (50 U/ml IL-3, 12.5 ng/ml GM-CSF, 100 ng/ml SCF, and 2 U/ml erythropoietin [Epo]) were added to the primary cell cultures together with the the third dose of oligodeoxynucleotides. IL-3 and GM-CSF were a generous gift of Genetics Institute, SCF was kindly provided by Immunex Corp., and Epo was from Amgen Inc. (Thousand Oaks, CA). Cells were further incubated for 16 h and plated in duplicate in 35-mm petri dishes without washing. Cells of established lines (10^5/ml) were incubated with or without growth factors, as indicated, in the presence of GAP sense or antisense oligodeoxynucleotides. Control groups were similarly cultured but without oligomers.

Colony Assays. Duplicate cultures were prepared in semisolid methylcellulose medium HCC 4230 (Terry Fox Labs., Vancouver, Canada) or plasma clot (27). Concentrations of growth factors and human AB serum during culture were fivefold lower than those used during the oligodeoxynucleotide treatment. In some cultures, no growth factors were added. For assay of myeloid colonies (CFU-GM), IL-3 and GM-CSF alone were used. BFU-E were induced in the presence of IL-3 and Epo or SCF plus Epo. Megakaryocytic colonies (CFU-Meg) were grown in the presence of IL-6 (Genetics Institute), IL-3, and Epo. Cultures were maintained for 10–12 d. Plates were scanned with an inverted microscope and the number of colonies (≥50 cells) and clusters (8–40 cells) was determined. Myeloid colonies were identified by visual inspection and erythroid colonies were counted after benzidine staining. Immunofluores-

cence analysis (36) with a glycoprotein IIb/IIIa–specific mAb (37) identified CFU-Meg. Different oligodeoxynucleotide preparations, several bone marrow donors, and CML patients, and the same batches of human AB serum and HCC 4230 medium were used.

Stimulation of GAP Expression by Growth Factors. Cells (10^6/ml IMDM supplemented with L-glutamine and 0.1% BSA) and growth factors were added at time 0. Cells used for measurement of GAP mRNA levels were incubated (10^5 cells/100 μl/well) in 96-well culture plates (Falcon, Becton Dickinson & Co., Lincoln Park, NJ). For protein studies, 10^6 cells/ml were incubated in 35-mm petri dishes (Nunc, Inc., Naperville, IL). Cells were collected and analyzed at the indicated times after adding the growth factors.

RT-PCR. Cells were collected separately from each group and total RNA was extracted (38). RNA from each group was divided into two portions. One sample was reverse transcribed using 400 U of Moloney murine leukemia virus reverse-transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and 0.1 μg of 3'-end primer of GAP for 1 h at 37°C. The second sample was reverse transcribed using the β-actin 3' primer. Resulting cDNA fragments were amplified with 5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in the presence of 5' primer of either GAP or β-actin generating 215- and 209-bp fragments of GAP and β-actin, respectively, during 50 cycles of PCR (39). Reaction products were electrophoresed, transferred and hybridized as described (27), using the appropriate probes.

Immunoprecipitation and Western Blotting. Cells (1–2 × 10^6) were solubilized in lysis buffer containing 10 mM Hepes, pH 7.5, 0.15 M NaCl, 10% glycerol, 10 μg/ml each of leupeptin and aprotinin (Sigma Chemical Co.), 1 mM PMSF, 1 mM Na_3VO_4, 1% NP-40, and 5 mM EDTA. Postnuclear lysates were immunoprecipitated for 2 h at 4°C with 5 μl of anti–human GAP rabbit serum (UBI, Inc., Lake Placid, NY) coupled to protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ). Beads were washed twice with lysis buffer and twice with 10 mM Hepes, 0.15 mM NaCl, 0.2% NP-40 solution, and eluted in sample buffer. Immunoprecipitated proteins or total cell lysates were separated on 7.5% SDS-PAGE and transferred to nitrocellulose (MCI, Westboro, MA). Filters were blocked with 0.5% gelatin in TBS and incubated with anti–human GAP rabbit serum, anti–human heat shock protein (HSP 72/73) murine monoclonal IgG2a (Oncogene Science Inc., Uniondale, NY), or anti-*abl* antiserum (40) overnight at 4°C. Filters were washed five times with 0.25% Tween, 0.25% NP-40 in TBS buffer. Proteins in the filters were detected using ^{125}I-Protein A (ICN Biomedicals, Costa Mesa, CA) or horseradish-peroxidase (HRP)-labeled anti–rabbit sera and enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Determination of GTP-bound $p^{21}RAS$. Cells (0.5 × 10^6/ml) were maintained for 18 h at 37°C in serum- and growth factor–free medium (IMDM supplemented with 0.5% dialyzed BSA and L-glutamine), washed, resuspended in phosphate-free medium, and labeled with ^{32}P-orthophosphate (NEN Research Products, DuPont, Boston, MA) for 3 h at 37°C. Levels of GTP/GDP-bound $p21^{ras}$ were determined as described (41) in 10^7 cells stimulated or not with IL-3 (10 U/ml in IMDM plus 0.1% BSA and L-glutamine).

Assay of p120 GAP Activity. Serum-starved MO7 cells (10^7/sample) were treated with IL-3 for the indicated times and assayed for GAP activity using purified $p21^{ras}$ (generous gift of Dr. F. McCormick, Onyx Pharmaceuticals, Richmond, CA) bound to α-[^{32}P]GTP as described (41). As indicated, GTPase promoting activity was also examined after immunoprecipitation of p120 GAP with anti-GAP serum or in the presence of *n*-dodecyl-β-D-mannoside (1 mM) which specifically inhibits the GTPase stimulating activity of NF-1 protein (42).

Results

Effect of GAP Antisense Oligodeoxynucleotides on Normal Hematopoietic Colony Formation. A-T-MNC were exposed to GAP oligodeoxynucleotides (160 + 80 + 80 μg/ml) and plated under optimal culture conditions for the growth of specific progenitor cell subsets. CFU-GM were grown in the presence of IL-3 or GM-CSF. BFU-E were expanded with IL-3 + Epo or SCF + Epo. CFU-Meg growing in the presence of IL-6 + IL-3 + Epo were identified by mAb immunofluorescence staining. As compared to controls, treatment with sense oligodeoxynucleotides did not reduce significantly the number of colonies derived from CFU-GM progenitors in the presence of IL-3 or GM-CSF (16.8 or 9.6% inhibition), from BFU-E progenitors in the presence of IL-3 + Epo or SCF + Epo (3.8 or 7.6% inhibition), or CFU-Meg progenitors (4.6% inhibition) (Table 1). By contrast, GAP antisense treatment caused significant inhibition of colony

Table 1. *Effect of GAP Sense and Antisense Oligodeoxynucleotides on In Vitro Colony Formation from Normal A-T-MNC*

		Number of colonies/plate			
Colonies	Inducer	Control	Sense	Antisense	Percent inhibition
					%
CFU-GM	IL-3	221.3 ± 14.4*	184.1 ± 32.0	93.9 ± 17.1	49.0 (p <0.001)‡
	GM-CSF	238.8 ± 42.3	215.8 ± 35.3	61.3 ± 20.8	71.6 (p <0.001)
BFU-E	IL-3 + Epo	156.0 ± 24.8	150.0 ± 8.0	72.5 ± 6.9	51.7 (p <0.001)
	SCF + Epo	36.8 ± 6.8	39.6 ± 6.8	11.7 ± 6.2	70.5 (p <0.001)
CFU-Meg	IL-6 + IL-3 + Epo	54.5 ± 13.2	52.0 ± 10.5	19.2 ± 9.7	63.1 (p <0.001)

* Values are mean ± SD from duplicate cultures from six to eight separate experiments from two to four different donors.
‡ Inhibition of colony formation by GAP antisense oligodeoxynucleotides in comparison with sense-treated groups. In parentheses, statistical significance (Student's t test).

formation by CFU-GM (49.0–71.6%), BFU-E (51.7–70.5%), and CFU-Meg (63.1%) as compared to sense-treated groups. The inhibitory effect was weak at lower concentration of GAP antisense oligodeoxynucleotides (80 + 40 + 40 μg/ml), and undetectable at the lowest concentration used (40 + 20 + 20 μg/ml).

To analyze the effect of GAP antisense oligodeoxynucleotides on the growth of more homogeneous, less mature populations of hematopoietic progenitors, colony assays were performed using CD34$^+$ MNC isolated from A-T-MNC (Table 2). Treatment of cells with sense oligodeoxynucleotides (160 + 80 + 80 μg/ml) inhibited colony growth stimulated with IL-3, GM-CSF, IL-3 + Epo, SCF + Epo, or IL-6 + IL-3 + Epo by only 14.3, 16.1, 7.7, 3.0, and 11.1% as compared with untreated cultures. By contrast, GAP antisense oligodeoxynucleotide treatment efficiently decreased the number of CFU-GM colonies induced by IL-3 or GM-CSF (56.5 or 51.1% inhibition, respectively), BFU-E colonies induced by IL-3 + Epo or SCF + Epo (67.5 or 43.1% inhibition), and CFU-Meg colonies induced by IL-3, IL-6, and Epo (59.6% inhibition) as compared with values obtained with sense oligodeoxynucleotide treatment (Table 2).

Effect of GAP Antisense Oligodeoxynucleotides on Proliferation of Growth Factor–dependent (MO7) or –independent (NB4 and HL-60) Cell Lines. To analyze whether only growth factor–dependent or also growth factor–independent proliferation of hematopoietic cells is related to GAP expression, similar experiments were performed on the MO7 cell line, dependent on IL-3, GM-CSF or SCF for proliferation, and on the growth factor–independent NB4 and HL-60 promyelocytic cell lines. GAP mRNA expression, as detected by RT-PCR on RNA extracted from each cell type 72 h after the first dose of oligodeoxynucleotide was added, was equally downregulated in the presence of GAP antisense, but not GAP sense, in all three cell lines (Fig. 1, *A–E*). The number of viable MO7 cells after 4, 6, and 8 d of culture with GAP antisense oligodeoxynucleotides was significantly lower than that of control or sense oligodeoxynucleotides–treated cells in response to any of the three growth factors (Fig. 1, *A–C*), whereas that of NB4 (Fig. 1 *D*) or HL-60 cells (Fig. 1 *E*) was unchanged compared to controls. As in the case of colony formation from primary cells, inhibition of MO7 cell proliferation was dose dependent: lower concentrations (80 + 40 + 40 μg/ml) of GAP antisense oligodeoxynucleotides induced only about 40–50% inhibition of cell growth and the effect was almost undetectable at concentrations of 40 + 20 + 20 μg/ml.

Effect of GAP Antisense Oligodeoxynucleotides on the Proliferation of bcr/abl–dependent CML-BC Cells. The growth of Philadelphia[1] BV173 cells and of primary cells from CML-BC patients used in these studies was highly dependent on *bcr/abl* expression, as demonstrated by the fact that exposure of these cells to antisense oligodeoxynucleotides designed against the specific breakpoint junction identified in each sample results not only in downregulation of *bcr-abl* mRNA expression, but also in complete inhibition of growth, and even death, of BV173 cells in liquid culture, and in significant inhibition (61.5–100%) of colony formation from marrow cells of CML-BC patients (UPN 1–5) in semisolid culture (data not shown). In agreement with reports by others (43), we observed a physical association of p210$^{bcr/abl}$ with p120 GAP in these cells (Fig. 2), suggesting the possibility of a functional link between these two proteins. To test this possibility, we examined the effect of GAP antisense oligodeoxynucleotides (160 + 80 + 80 μg/ml) on proliferation of BV173 cells. The cell growth was completely inhibited and GAP, but not β-actin mRNA levels, were simultaneously downregulated, as detected by RT-PCR (Fig. 3). This effect was dose dependent (data not shown). Moreover, GAP antisense, but not sense, oligomers caused selective downregulation of the levels of p120 GAP protein by 50–67% (data from two independent experiments), but not those of HSP (Fig. 3),

Table 2. *Effect of GAP Sense and Antisense Oligodeoxynucleotides on In Vitro Colony Formation from Normal Bone Marrow CD34⁺ MNC*

Colonies	Inducer	Number of colonies/plate			
		Control	Sense	Antisense	Percent inhibition
					%
CFU-GM	IL-3	245.3 ± 31.9*	210.3 ± 38.4	91.5 ± 34.0	56.5 (p <0.001)‡
	GM-CSF	222.0 ± 24.2	186.2 ± 10.6	91.0 ± 7.1	51.5 (p <0.001)
BFU-E	IL-3 + Epo	194.5 ± 12.0	179.2 ± 17.5	58.2 ± 15.5	67.5 (p <0.001)
	SCF + Epo	73.0 ± 8.3	70.8 ± 16.2	40.3 ± 4.7	43.1 (p <0.001)
CFU-Meg	IL-6 + IL-3 + Epo	23.4 ± 4.9	20.8 ± 3.7	8.4 ± 2.4	59.6 (p <0.001)

* Values are mean ± SD from duplicate cultures from six to eight separate experiments from two to four different donors.
‡ Inhibition of colony formation by GAP antisense oligodeoxynucleotides in comparison with sense-treated groups. In parentheses, statistical significance (Student's t test).

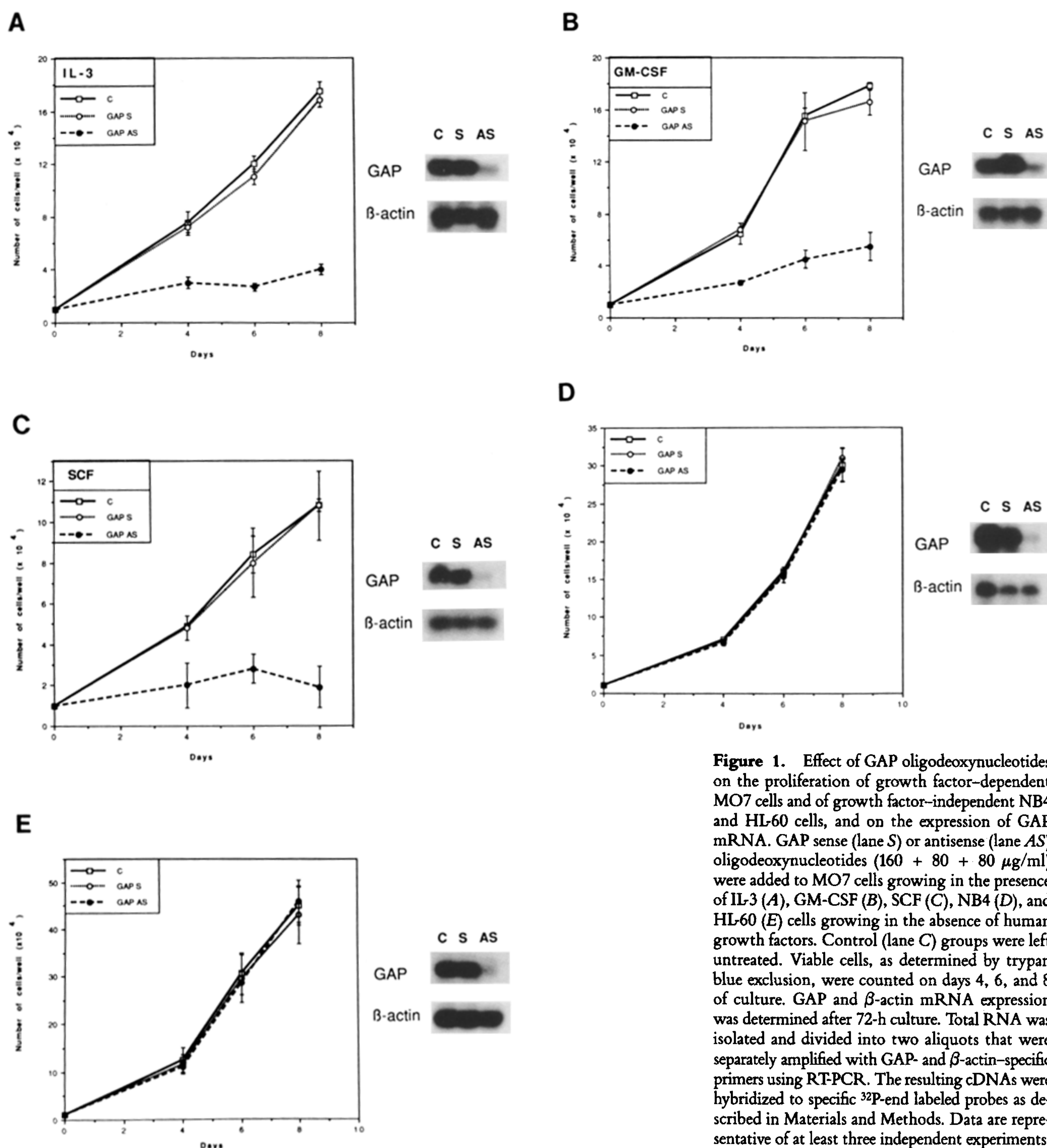

Figure 1. Effect of GAP oligodeoxynucleotides on the proliferation of growth factor–dependent MO7 cells and of growth factor–independent NB4 and HL-60 cells, and on the expression of GAP mRNA. GAP sense (lane *S*) or antisense (lane *AS*) oligodeoxynucleotides (160 + 80 + 80 μg/ml) were added to MO7 cells growing in the presence of IL-3 (*A*), GM-CSF (*B*), SCF (*C*), NB4 (*D*), and HL-60 (*E*) cells growing in the absence of human growth factors. Control (lane *C*) groups were left untreated. Viable cells, as determined by trypan blue exclusion, were counted on days 4, 6, and 8 of culture. GAP and β-actin mRNA expression was determined after 72-h culture. Total RNA was isolated and divided into two aliquots that were separately amplified with GAP- and β-actin–specific primers using RT-PCR. The resulting cDNAs were hybridized to specific ^{32}P-end labeled probes as described in Materials and Methods. Data are representative of at least three independent experiments.

as detected by Western blotting, further supporting that the effects of GAP antisense treatment described above are sequence specific.

CML-BC primary cells (UPN 1–5) were incubated with GAP antisense oligodeoxynucleotides under the same conditions as normal bone marrow cells, except that in some cases exogenous IL-3 was omitted from the cultures (Table 3). In one experiment performed on cells from patient UPN 1, CD34^{+} MNC were used. GAP sense oligodeoxynucleotides did not influence significantly the growth of CML-BC primary cells compared to that of control untreated cells (−3.5–11.0% inhibition). Both IL-3–dependent and IL-3–independent CML-BC colony formation was significantly inhibited by treatment with GAP antisense oligodeoxynucleo-

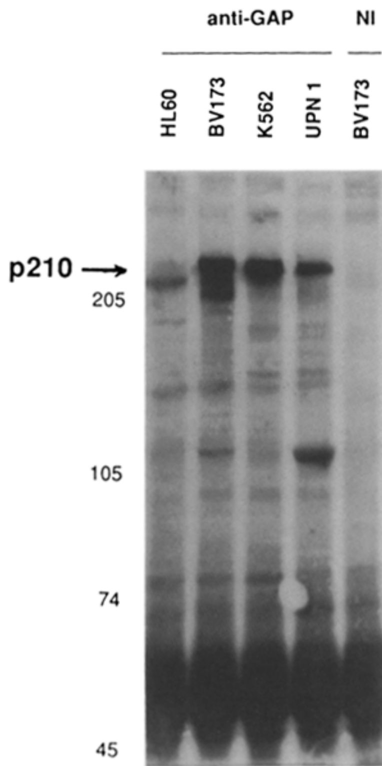

Figure 2. Association of $p210^{bcr/abl}$ and p120 GAP. Anti-GAP or nonimmune (*NI*) control sera were used for immunoprecipitation from the indicated cell lines or patient primary cells. The immunoprecipitates were resolved in 7.5% SDS-PAGE and transferred to nitrocellulose membrane. Anti-*abl* antibody was used for Western blot analysis of the transferred proteins.

tides (55.7–87.4% and 87.3–91.0% inhibition, respectively) in comparison to sense-treated groups. A 3-d incubation of CML-BC cells (UPN 1) in the presence of GAP antisense oligodeoxynucleotides (160 + 80 + 80 μg/ml) caused downregulation of GAP but not of β-actin mRNA expression (Fig. 4).

Effect of GAP Oligomers on p120 GAP Expression in Growth Factor–stimulated MO7 Cells. To examine the effect of growth factors on GAP mRNA levels, MO7 cells were incubated in serum-free medium for 12 h, after which IL-3 was added and total RNA and postnuclear cell lysates were prepared from an equal number of cells 0, 4, 8, 12, 24, and 48 h later. Increased expression of GAP mRNA, and protein levels (4–9-fold in three experiments), were detected by RT-PCR and Western blotting, respectively, starting 4 h after stimulation (Fig. 5, *A* and *B*). β-actin mRNA and HSP levels remained constant throughout the culture period. To determine whether the GAP antisense could inhibit IL-3–induced p120 GAP overexpression, MO7 cells were serum starved and IL-3 stimulated in the presence of GAP oligomers (160 + 80 + 80 μg/ml). Postnuclear cell lysates were collected from 10^6 cells after 48-h stimulation. GAP antisense, but not sense, oligomers inhibited IL-3–inducible GAP mRNA (data not shown) and p120 GAP expression by 67–85% (data from three experiments) in comparison to controls (Fig. 5 *C*).

$p21^{ras}$ Activity after IL-3 Stimulation. We determined the fraction of GTP-bound *RAS* in MO7 cells stimulated with IL-3. Only 3.3% of the $p21^{ras}$ was detected in the active GTP-bound form in MO7 cells incubated for 18 h in serum- and growth factor–free medium. This fraction increased to 10.7% after 5-min incubation with IL-3 (Fig. 6 *A*) and remained at levels (6.7%) lower than this, but significantly higher than those in control untreated cells, after 48-h culture in the presence of IL-3 (Fig. 6 *A*).

GTPase-promoting Activity in MO7 Cells after IL-3 Stimulation. Because GTPase promoting activity is involved in regulating the status of $p21^{ras}$ activation, we determined it in MO7 cells stimulated with IL-3. 15.6 ± 0.7, 24.2 ± 3.7, and 6.6 ± 4.4% of $p21^{ras}$ was in the GTP-bound form after incubation with total cells lysate obtained from serum- and growth factor–starved cells or cells obtained 5 min and 48 h after IL-3 stimulation, respectively (Fig. 6 *B*). Compared to untreated cells, GTPase-promoting activity was inhibited 5 min after IL-3 stimulation, but was even higher at 48 h. As

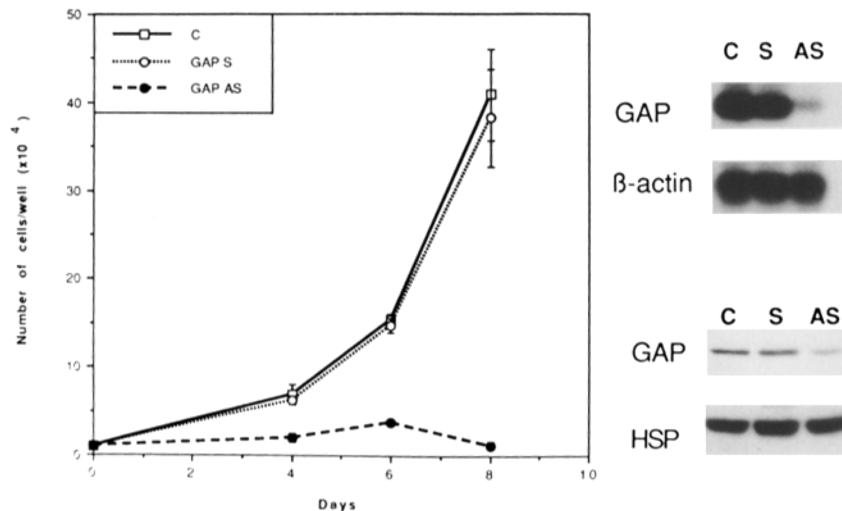

Figure 3. Effect of GAP oligodeoxynucleotides on the proliferation and GAP mRNA expression of *bcr/abl*-dependent BV173 cells. Growth of BV173 cells and levels of GAP and β-actin mRNA were determined as described in Fig. 1. The levels of GAP protein and HSP 72/73 were analyzed by Western blotting in postnuclear cell lysates obtained from cells after 72-h incubation with sense (*S*) or antisense (*AS*) oligomers. Control (*C*) cells were left untreated.

Table 3. *In Vitro CML-BC Cells Colony Formation in the Presence of GAP Sense and Antisense Oligodeoxynucleotides*

		Number of colonies/plate			
Patient	Inducer	Control	Sense	Antisense	Percent inhibition
					%
UPN1	IL-3	2,594.3 ± 261.1*	2,496.0 ± 338.0	548.5 ± 27.6	78.0 (p = 0.015)‡
	None	425.7 ± 65.5	378.7 ± 6.7	34.0 ± 7.8	91.0 (p <0.001)
UPN1/CD34+	IL-3	265.5 ± 23.3	233.0 ± 36.8	28.0 ± 4.2	87.4 (p = 0.006)
UPN2	IL-3	1,778.0 ± 76.4	1,512.8 ± 280.5	322.0 ± 5.7	78.7 (p = 0.005)
	None	81.5 ± 4.9	74.8 ± 6.3	9.5 ± 0.7	87.3 (p <0.001)
UPN3	IL-3	198.5 ± 12.7	191.0 ± 7.1	85.0 ± 11.3	55.7 (p = 0.008)
UPN4	IL-3	1,129.0 ± 76.4	1,168.8 ± 124.7	183.3 ± 34.6	83.7(p <0.001)
	None	1,144.5 ± 170.4	960.3 ± 90.7	93.5 ± 2.1	90.3 (p <0.001)
UPN5	IL-3	27.0 ± 5.7	23.0 ± 4.2	4.0 ± 1.4	82.67 (p = 0.027)

* Cells (10^5 A-T-MNC or, where indicated, 10^4 CD34+ cells) were cultured in the presence or absence of IL-3. Results are expressed as mean ± SD from quadruplicate determination in two to four independent experiments.
‡ Inhibition of colony formation by GAP antisense oligodeoxynucleotides in comparison with sense-treated groups. In parentheses, statistical significance (Student's t test).

shown in Fig. 6 *B* inset, ~45% of this activity was due to p120 GAP, as revealed by performing the assay with GAP-depleted lysates, the remaining activity was most likely due to NF-1 as revealed by a 55% inhibition of GTPase promoting activity when the assay was performed in the presence of the specific NF-1 inhibitor *n*-dodecyl-β-D-mannoside.

Discussion

A powerful experimental approach to define the function of a gene is to specifically inhibit its in vivo activity. In mammalian cells, this can be accomplished with the antisense strategy (44). Using this approach to dissect the role of individual genes in signal transduction pathways, we observed that the N-*ras* protooncogene is important in the growth of normal human hemopoietic cells (27). p21^{ras}, like other GTP-binding proteins, is activated by the replacement of GDP by GTP, a process that is catalyzed by a guanine nucleotide–releasing factor (16). The active p21^{ras}-GTP–bound form is inactivated by an intrinsic GTPase activity that is catalyzed by the COOH-terminal domain of GAP (45). Increasing evidence suggests that, in addition to its interaction with p21^{ras} via the COOH-terminal domain, GAP also interacts with phosphoproteins with tyrosine kinase activity such as the

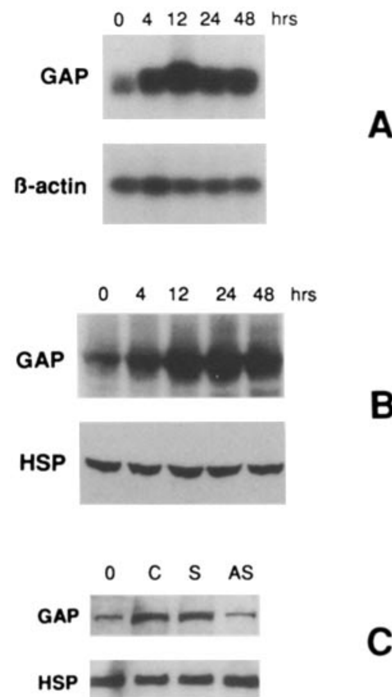

Figure 5. Expression of GAP in IL-3–stimulated and oligodeoxynucleotide-treated MO7 cells. Cells were incubated with IL-3 (*A* and *B*) or IL-3 plus oligodeoxynucleotides (*C*). Total RNA and cell lysates were prepared at the indicated times (*hrs*). (*A*) RT-PCR analysis of GAP and β-actin mRNA levels; (*B* and *C*) GAP protein and HSP 72/73 analysis. GAP protein expression was analyzed by Western blotting of total cell lysates using anti-GAP serum (7.5% SDS-PAGE). As control of the amount of proteins loaded per lane, the same filter was sequentially hybridized with anti-heat shock protein (HSP 72/73) mAb. Densitometric measurements of the bands were performed using an Ultro Scan XL (Pharmacia LKB Biotechnology) apparatus.

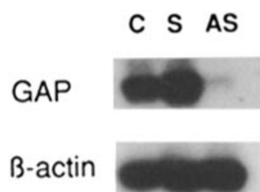

Figure 4. Expression of GAP and β-actin mRNA in CML-BC cells (UPN 1) exposed to GAP oligodeoxynucleotides. GAP and β-actin mRNA levels were analyzed as described in Fig. 1.

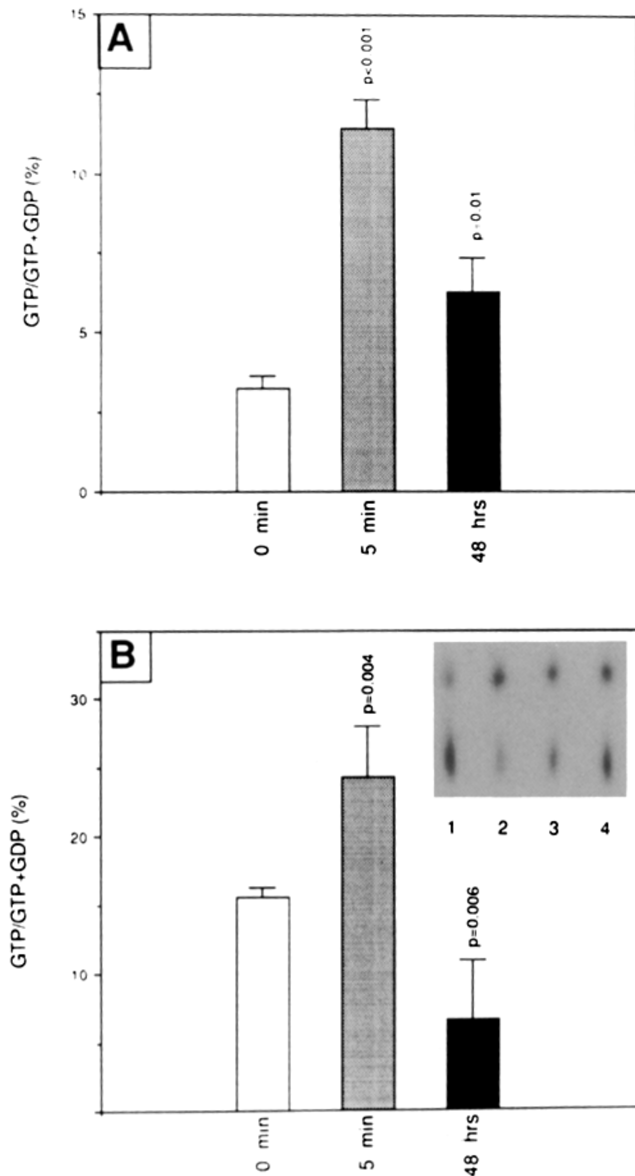

Figure 6. Activation of p21^{ras} and GTPase promoting activity in IL-3-stimulated MO7 cells. Serum and growth factor–starved cells were left unstimulated or were stimulated with IL-3 for 5 min or 48 h. Activation status of p21^{ras} (*A*) and GTPase promoting activity (*B*) were examined at each time point. Bars represent values (mean ± SD) from three (*A*) and two (*B*) independent experiments (each performed in duplicate) of GTP-bound p21^{ras} over total (GTP- and GDP-bound). *P* values (Student's *t* test) indicate the level of statistical significance compared to control untreated group. The percentage of p120 GAP and NF-1 dependent GTPase promoting activity (*B, inset*) was established after incubation of p21^{ras}-GTP complex (lane *1*) with total cell lysate from MO7 cells stimulated with IL-3 for 48 h (lane *2*) from which the p120 GAP or NF-1 activity were removed by immunoprecipitation with anti-GAP serum (lane *3*) or incubation in the presence of *n*-dodecyl *β*-D-mannoside (lane *4*).

PDGF and EGF receptors, *src*, *bcr-abl*, p190, p62, and p56^{lck}, via two SH2 domains in the NH_2-terminal region (43, 46–50) and with cytoskeleton via one SH3 domain (51). Those observations led to the hypothesis that GAP can function both as signal terminator (catalysis of p21^{ras} intrinsic GTPase activity which impairs p21^{ras} signal-transducing ability) and as signal transducer (linking p21^{ras} with tyrosine kinases) (52). The observation that induction of gene expression by GAP SH2-SH3 domains depends on p21^{ras} activity (53) suggests that p21^{ras}-GTP induces conformational changes in GAP which allow the SH2-SH3 domains to function.

To assess the functional requirement of GAP as a signal transducer in normal and leukemic cells, we examined the proliferation and colony-forming ability of these cells in the presence of GAP antisense oligodeoxynucleotides. Colony formation by myeloid, erythroid, and megakaryocytic progenitors isolated from normal bone marrow cells (A-T-MNC or CD34$^+$ cells) required GAP expression as did the growth of the IL-3–dependent AML MO7 cell line, of the Philadelphia[1] *bcr/abl* tyrosine kinase–dependent BV173 line, and of CML primary cells. Because proliferation of the growth factor–independent NB-4 and HL-60 cell lines is not affected by downregulation of GAP expression resulting from treatment with antisense oligodeoxynucleotides, it appears that GAP expression is required for proliferation in cells that are growth factor–dependent or, as in the case of Philadelphia[1] cells, when the oncogenic activation (formation of *bcr-abl* genes) replaces growth factor requirements.

The growth and differentiation of hematopoietic progenitors induced by several growth factors (IL-3, GM-CSF, SCF, and Epo) is accompanied by activation of p21^{ras} (9, 10, 54) and phosphorylation of several target proteins (7, 55). *Bcr/abl* tyrosine kinase activates p21^{ras} (15), induces protein phosphorylation (55, 56), and is required for proliferation and survival of CML cells (57, 58). Thus, it is conceivable that GAP joins p21^{ras} (via its COOH terminus) and tyrosine kinases (via its NH_2 terminus) in the signal transduction pathway, while maintaining its ability to convert p21^{ras} to the inactive GDP-bound form, thus terminating the signal-transducing stimulus. By inhibiting GAP expression, the link between p21^{ras}-GTP and tyrosine kinases may have been disrupted.

Because the levels of GAP expression appeared to be important in determining its effect on cell growth (59), we examined the expression of GAP after growth factor stimulation. Our experiments show that IL-3 upregulates GAP mRNA as well as protein levels. A similar phenomenon was observed also in CD34$^+$ bone marrow cells after stimulation with IL-3, GM-CSF or SCF (data not shown). Inhibition of growth factor–stimulated cellular proliferation by GAP antisense oligomers correlates with their ability to block the growth factor–induced GAP overexpression, which strongly suggests that increased levels of GAP expression could be important for cell growth. The increase of GAP protein level correlated well with higher GTPase promoting activity in MO7 cells (45% GAP dependent, 55% NF-1 dependent) after long-term (48 h) stimulation with IL-3. In contrast, short-term (5 min) stimulation induced a decrease of this activity. Similar short-term changes in GTPase promoting activity were observed in cells stimulated with Epo (54) and EGF (56). The changes in GTPase promoting activity in lysates of IL-3–stimulated MO7 cells were partially in agreement with the p21^{ras} GTP-GDP status. The p21^{ras}-GTP–bound fraction

was increased ~threefold shortly (5 min) after IL-3 addition and was still ~twofold higher after long-term (48 h) incubation in the presence of the cytokine. The status of p21^{ras} activation is the result of stimulatory (guanine-nucleotide releasing factor [GNRF]) and inhibitory (GAP, NF-1) control mechanisms. Shortly after cytokine stimulation, p21^{ras} becomes activated because of the increased GNRF activity (16) and the decreased GTPase promoting activity (54, and our observations). By comparison, in MO7 cells exposed for a much longer time (48 h) to IL-3, the levels of active GTP-bound p21^{ras} are reduced, perhaps reflecting a steady-state situation in which stimulatory mechanisms (GNRF) are balanced by the inhibitory ones (GTPase promoting activity of GAP and NF-1). The increase of GAP levels after long-term treatment of MO7 cells with IL-3 correlates in part with an increase in the GTPase promoting activity. However, some of this activity is mediated by NF-1, thereby making it possible for GAP to function as a signal transducer. Our findings in normal and leukemic hematopoiesis provide direct evidence for such a role.

This work was supported in part by National Institutes of Health grants CA-46782, CA-37155, and CA-45286 and American Cancer Society grants CH-492 and CH-455A. T. Skorski is supported by a fellowship from the Cancer Research Foundation of America. B. Calabretta is a scholar of the Leukemia Society of America.

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Received for publication 7 June 1993 and in revised form 13 September 1993.

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