Raf-1 Protein Is Required for Growth Factor–induced Proliferation of Hematopoietic Cells

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Summary

Raf-1 is a 74-kD serine/threonine kinase located in the cell cytoplasm that is activated by phosphorylation in cells stimulated with a variety of mitogens and growth factors, including hematopoietic growth factors. Using c-*raf* antisense oligonucleotides to block Raf-1 expression, we have established that Raf-1 is required for hematopoietic growth factor–induced proliferation of murine cell lines stimulated by growth factors whose receptors are members of several different structural classes: (*a*) the hematopoietin receptor family, including interleukin (IL)-2, IL-3, IL-4, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor (GM-CSF), and erythropoietin; (*b*) the tyrosine kinase receptor class, including Steel factor and CSF-1; and (*c*) IL-6, leukemia inhibitory factor, and oncostatin M, whose receptors include the gp130 receptor subunit. Although results of previous experiments had suggested that IL-4 does not phosphorylate or activate the Raf-1 kinase, c-*raf* antisense oligonucleotides inhibited IL-4–induced proliferation of both myeloid and T cell lines, and IL-4 activated Raf-1 kinase activity in an IL-4–dependent myeloid cell line. In colony assays, c-*raf* antisense oligonucleotides completely inhibited colony formation of unseparated normal murine bone marrow cells stimulated with either IL-3 or CSF-1 and partially inhibited cells stimulated with GM-CSF. In addition, c-*raf* antisense oligonucleotides completely inhibited both IL-3– and GM-CSF–induced colony formation of $CD34^+$ purified human progenitors stimulated with these same growth factors. Thus, Raf-1 is required for growth factor–induced proliferation of leukemic murine progenitor cell lines and normal murine and human bone marrow–derived progenitor cells regardless of the growth factor used to stimulate cell growth.

The proliferation and differentiation of hematopoietic progenitor cells are dependent on the activation of cell surface receptors by a family of extracellular glycoproteins known as hematopoietic growth factors (1). Binding of hematopoietic growth factors to their cell surface receptors induces oligo- or heterodimerization of receptor subunits and activation of a tyrosine phosphorylation–dependent cascade of events leading to the induction of DNA synthesis and immediate early gene expression in the nucleus (2–4). Hematopoietic growth factors activate associated or intrinsic tyrosine kinases depending on the structural characteristics of the receptor. Members of the hematopoietin receptor family do not contain intrinsic tyrosine kinase activity, but they activate associated tyrosine kinases (3, 4), whereas members of the tyrosine kinase receptor class contain an intracellular tyrosine kinase in the cytoplasmic domain of the receptor (2). Structural differences between the various classes of hematopoietic growth factor receptors suggest that these receptors may activate different signal transduction pathways (2, 5–7). However, it has been demonstrated that ligand stimulation of different classes of growth factor receptors induces phosphorylation of a similar although not identical set of intracellular proteins (3, 4).

One of these proteins, Raf-1, a 74-kD serine/threonine kinase located in the cell cytoplasm, is activated by phosphorylation after mitogen or growth factor stimulation in a variety of cell types, including hematopoietic cells (8–15). This property suggests that Raf-1 may play a role in hematopoietic cell growth.

Biochemical studies demonstrated that the Raf-1 kinase is activated by phosphorylation in growth factor–dependent cell lines stimulated with IL-2, IL-3, GM-CSF, erythropoietin

(EPO)[1], G-CSF, CSF-1, and Steel factor (SLF) (16–20). However, only one of these studies investigated the biological effects of Raf-1 activation on cell growth (18). In addition, the role of Raf-1 in normal hematopoiesis is unknown. Therefore, to determine the requirement for Raf-1 in growth factor–regulated proliferation and differentiation of hematopoietic cells, we studied the effect of inhibiting Raf-1 expression using c-*raf* antisense oligonucleotides on (*a*) growth factor–induced proliferation of myeloid progenitor cell lines stimulated with cytokines that activate several different classes of hematopoietic growth factor receptors, and (*b*) CSF-induced colony formation of normal murine and human bone marrow cells.

This study demonstrates that Raf-1 is required to transduce the growth factor–induced proliferative signal in factor-dependent leukemic cell lines for all known classes of hematopoietic growth factor receptors and that it is required for CSF-induced colony formation of normal murine and human bone marrow–derived progenitors.

Materials and Methods

Cell Lines. FDCP-1 (21) and 32D-Cl3 (22) cells are IL-3–dependent murine myeloid progenitor cell lines derived from long-term in vitro mouse bone marrow cultures; 32D-c-*fms* cells are 32D-Cl3 cells that express a transfected human CSF-1 receptor (23). The NFS-60, DA-3, and DA-1A cell lines (24) are IL-3–dependent murine myeloid cell progenitor cell lines derived from the preleukemic spleens of mice infected with murine leukemia viruses. HCD-57 is an EPO-dependent erythroleukemia cell line derived from a newborn mouse infected with Friend murine leukemia virus (25). CTLL-2 is an IL-2–dependent T cell line derived from long-term cultures of PBLs stimulated with PHA (26). All cell lines were maintained in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 3 mg/ml L-glutamine (complete medium), and the appropriate growth factor. Media for maintaining HCD-57 cells included 5×10^{-5} M β-mercaptoethanol.

Normal Bone Marrow Cells. Normal murine bone marrow cells were aspirated from BALB/c mouse femurs with IMDM and layered on lymphocyte separation medium (Organon Teknika Corp., Durham, NC) to obtain light density cells. Lin^- cells were selected using previously published techniques (27). Briefly, unseparated bone marrow cells were incubated with RA3-6B2 (B220 antigen), RA3-8C5 (both are gifts from R. Coffman, DNAX Corp., Palo Alto, CA), Mac-1 (Boehringer Mannheim Biochemicals, Indianapolis, IN), Lyt-2 (CD8), and L3T4 (CD4) (Becton Dickinson, Rochelle Park, NJ) antibodies, which recognize myeloid- and lymphoid-specific cell surface antigens, for 30 min at 4°C. Cells were then washed twice and incubated with anti-rat IgG–coated magnetic beads (Dynal Corp., Oslo, Norway) at a bead/cell ratio of 40:1 for 30 min at 4°C. Cells were then magnetically separated with a magnetic particle concentrator (Dynal Corp.), and the Lin^- cells were recovered in the supernatant and resuspended in complete IMDM. Human bone marrow cells were obtained by aspiration from healthy donors after informed consent. The low density mononuclear cells were isolated from the interphase after Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) gradient centrifugation, washed twice in IMDM, and suspended in IMDM supplemented with 25% FCS, 1% detoxified BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 3 mg/ml L-glutamine. $CD34^+$ cells were obtained by positive selection using previously published techniques (28). Briefly, magnetic beads (Dynabead M-450 CD34; Dynal Corp.) with CD34-specific BL-3C5 mAbs attached to them were added to cells at a bead/cell ratio of 1:1 and an anti-Fab antiserum (10 μl/1 × 10^7). Detachabead (Dynal Corp.) was used for detachment of beads from positively selected cells. $CD34^+$ cells were recovered by magnetically separating the beads using a magnetic particle concentrator (Dynal Corp.) and suspended in complete medium.

Growth Factors. Purified recombinant murine IL-3 and murine GM-CSF were purchased from PeproTech (Rocky Hill, NJ). Purified recombinant murine G-CSF was supplied by L. Souza (Amgen Corp., Thousand Oaks, CA), and recombinant murine EPO was purchased from Amgen Corp. Purified recombinant murine CSF-1 was a gift from M. Geier (Cetus Corp., now Chiron Corp., Mountain View, CA), and IL-2 was purchased from Cetus Corp. Murine rIL-4 was purchased from Genzyme Corp. (Boston, MA). Recombinant murine IL-6, leukemia inhibitory factor (LIF), and oncostatin M (OSM) were purchased from PeproTech. Murine SLF and recombinant human IL-3 were a gift from S. Gillis (Immunex Corp., Seattle, WA). Recombinant human GM-CSF was a gift from I. McNiece (Amgen Corp.).

Synthesis of Oligonucleotides. Phosphorothioate antisense (5′-TCC-CTGTATGTGCTCCAT-3′), sense (5′-ATGGAGCACATACAG-GGA-3′), and nonsense (5′-TTTTTGCACCAGCTTGCC-3′) oligodeoxyribonucleotides were synthesized by the phosphoramadite method using standard procedures on an automated synthesizer (model 380-B; Applied Biosystems, Inc., Foster City, CA) (18). Sulfurization was performed after synthesis of the oligonucleotides using the H-phosphonate method (29). Oligonucleotides were purified by polyacrylamide gel electrophoresis and TLC. Purified oligomers dissolved in ammoniated water were concentrated in a Speed Vac concentrator (Savant Inc., Farmingdale, NY) and were resuspended in sterile H_2O at a 100 μM concentration.

[3H]Thymidine Incorporation Assay. Growth factor–dependent cells (5×10^3) were washed free of growth factor and then seeded in 100 μl of RPMI plus 10% FCS in 96-well plates and preincubated overnight (12–15 h) with one-half of the final concentration of oligonucleotides at 37°C, 5% CO_2. All cell lines were treated with a 7.5 μM concentration of c-*raf* antisense, sense, or nonsense oligonucleotides with the exception of HCD-57 cells, which were treated with a 3.5 μM concentration of oligonucleotides. Cells growing in media alone or in media plus growth factors were used as controls. The next day, the remaining aliquot of oligonucleotides was added, and cells were stimulated with the following growth factors: FDCP-1 cells were stimulated with IL-3 (30 ng/ml), IL-4 (40 ng/ml), or SLF (50 ng/ml); DA-3 cells were stimulated with GM-CSF (20 ng/ml); 32D-Cl3 cells were stimulated with G-CSF (50 ng/ml); 32D-CSF-1 cells were stimulated with CSF-1 (50 ng/ml); and DA-1A cells were stimulated with IL-6 (50 ng/ml), LIF (200 ng/ml), or OSM (200 ng/ml). CTLL-2 T cells were stimulated with IL-2 (100 U/ml) or IL-4 (40 ng/ml). Cells were grown for 42 h at 37°C, 5% CO_2 and pulsed with 1 μCi of [3H]thymidine for 6–8 h. Cells were harvested onto glass filters, and [3H]thymidine incorporation was measured by scintillation counting.

Soft Agar Colony Assay. A modification of the method of Stanley et al. (30) was used to measure colony formation of bone marrow cells. Briefly, 2×10^5 unseparated murine or human bone marrow cells or 2×10^4 purified progenitors (Lin^- or $CD34^+$) were sus-

[1] *Abbreviations used in this paper:* EPO, erythropoietin; GST, glutathione-*S*-transferase; INT, nitrophenyl-*s*-phenyltetrazolium chloride; LIF, leukemia inhibitory factor; MBP, myelin basic protein; MEK, microtubule-associated protein kinase kinase; SLF, Steel factor.

pended in 100 μl of complete medium plus growth factors in 15-ml tubes. Murine bone marrow cells were stimulated with recombinant murine IL-3 (30 ng/ml), GM-CSF (50 ng/ml), or CSF-1 (50 ng/ml), and human bone marrow cells were stimulated with recombinant human IL-3 (30 ng/ml) or GM-CCSF (50 ng/ml). One-half of the final concentration of oligonucleotides was added to each tube, and cells were incubated overnight for 12–15 h at 37°C, 5% CO_2. The next day, the remaining oligonucleotides were added to each 15-ml tube, and cells were allowed to sit at room temperature for 30 min. Complete medium supplemented with growth factors at previously indicated concentrations was added to each 15-ml tube to give a final volume of 1 ml per plate, and cells were plated in 0.3% Seaplaque agarose (FMC Bioproducts, Rockland, ME) in 35-mm Lux petri dishes (Miles Scientific, Naperville, IL). Cell viability was assessed by trypan blue exclusion counting before plating, and all samples contained >90% viable cells. Plates were incubated in a fully humidified incubator at 37°C, 5% CO_2. Colonies containing >20 cells were scored on day 7 for unseparated and Lin^- purified murine bone marrow cells and on day 14 for unseparated or $CD34^+$ purified human bone marrow cells. For photography, colonies were stained with 0.1% 2-iodophenyl-3,4-nitrophenyl-*s*-phenyltetrazolium chloride (INT; Aldrich Chemical Co., Milwaukee, WI) for 3 d at 37°C.

Western Blot Protein Analysis. Growth factor–starved IL-3–dependent FDCP-1 cells were seeded at 5×10^5 cells in 1 ml in a 24-well plate and treated with either sense or antisense oligonucleotides (7.5 μM) as described for the [^{3}H]thymidine assay. Untreated FDCP-1 cells stimulated with IL-3 were used for the media control. Cells were harvested 36 h after the addition of IL-3 and lysed in 100 μl of RIPA buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1% Triton X-100, 0.5% desoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 25 mM glycerophosphate). Insoluble material was removed by centrifugation at 4°C for 30 min at 12,000 *g*, and protein concentrations were determined (protein assay; Bio Rad Laboratories, Hercules, CA). Whole protein lysates were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blots were blocked with 5% gelatin in TBST buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.5% Triton X-100) containing 5% FCS for 10 min and then incubated for 1 h at 4°C with polyclonal antisera raised against synthetic peptides corresponding to COOH-terminal amino acid sequences of either Raf-1 (anti-SP63 antibody) (31) or A-Raf (32). 100 μg of protein from untreated cells was analyzed in the absence and presence of the competing peptide (10 μg/ml) using anti-Raf (SP63) antisera, and 45 μg of protein from oligonucleotide-treated cells was analyzed for expression of Raf-1 using anti-Raf (SP63) antisera. A-Raf protein expression was detected by probing a parallel blot with anti–A-Raf antisera. After incubation with the antisera, blots were washed three times for 5 min with TBST buffer containing 5% FCS and then incubated with a secondary antibody (goat anti–rabbit IgG) conjugated to alkaline phosphatase for 30 min at room temperature. Protein bands were detected using the ECL system (Amersham Corp., Arlington Heights, IL).

Immunocomplex Kinase and Phosphorylation-induced Shift Assay. FDCP-1 cells (2×10^6) maintained in IL-4 (40 ng/ml) were grown to confluency in a 75-cm^2 flask and then washed free of growth factor and starved overnight (12–15 h) in RPMI plus 10% FCS. Cells were stimulated with IL-4 for 5–30 min and then immediately lysed in RIPA buffer. Protein concentrations were determined using the Bio Rad protein assay. Raf-1 proteins were immunoprecipitated from 1.5 mg of total protein by incubating lysates with anti-Raf (SP63) antisera and protein A agarose for 3 h at 4°C. For the in vitro kinase reaction, immunoprecipitated Raf-1 protein was washed three times with TBST and two times with kinase buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 25 mM DTT, 5 mM $MnCl_2$, 5 mM $MgCl_2$), and the pellets were resuspended in 30 μl of kinase buffer containing 10 μM ATP and 20 μCi of γ-^{32}ATP. As substrates, we added either myelin basic protein (MBP; Sigma Chemical Co., St. Louis, MO) or a fusion of glutathione-*S*-transferase and kinase-dead, microtubule-associated protein kinase kinase purified over glutathione agarose (GST-MEK^-). Samples were incubated for 30 min at room temperature. The reaction was stopped with Laemmli buffer, and proteins were resolved on a 4–20% SDS gel. MPB bands were visualized by autoradiography. To evaluate Raf-1 for a phosphorylation-induced shift in molecular weight, proteins were resolved on a 7.5% SDS–polyacrylamide gel and analyzed by Western blot analysis using anti-Raf (SP63) antisera as described above.

Results

c-raf Antisense Oligonucleotides Inhibit Growth Factor–induced Proliferation of Factor-dependent Cell Lines. To determine the role of Raf-1 in hematopoietic cell growth, we first evaluated the effect of *c-raf* antisense oligonucleotides on growth factor–induced proliferation of factor-dependent cell lines. Phosphorothioate deoxyoligonucleotides containing 18-bp sequences either complementary (antisense) or identical (sense) to the region surrounding translation start site of the *c-raf* gene and 17-bp oligomers containing randomly generated sequences (nonsense) with the same overall base composition as the antisense oligomer were synthesized using standard phosphoramadite chemistry (29). To determine the dose response of the oligonucleotides, we first evaluated the effect of the oligonucleotides on proliferation of FDCP-1 cells stimulated with IL-3 using the [^{3}H]thymidine incorporation assay. *c-raf* antisense oligonucleotides inhibited IL-3–induced proliferation of FDCP-1 cells in a dose-dependent manner (data not shown), with maximum inhibition (>98%) achieved at a 7.5 μM concentration of antisense oligonucleotides; a 7.5 μM concentration of sense and nonsense oligonucleotides had no significant effect on IL-3–induced proliferation (Fig. 1 *A*). In both sense and antisense oligonucleotide–treated cultures, cell viability assessed by trypan blue exclusion counting was >85% for up to 5 d, indicating that at this concentration the oligonucleotides were not toxic to the cells (data not shown).

In addition to IL-3, we examined the effect of the oligonucleotides on other CSFs that use hematopoietin receptors including GM-CSF, G-CSF, and EPO (6). Similar to their effect on IL-3–induced proliferation in FDCP-1 cells (>98% inhibition), *c-raf* antisense oligonucleotides inhibited GM-CSF–induced proliferation of DA-3 cells, G-CSF–induced proliferation of 32D-Cl3 cells, and EPO-induced proliferation of HCD-57 cells by 95–100%, whereas sense oligonucleotide–treated cells were not affected (Fig. 1 *A*).

A subclass of the hematopoietin receptor family that includes the receptors for IL-6, LIF, and OSM transduces proliferative signals through dimerization of a ligand-binding subunit with the gp130 subunit, which is the signal-transducing component of these receptors (7, 33, 34). To determine the requirement for Raf-1 in gp130-mediated signal transduction,

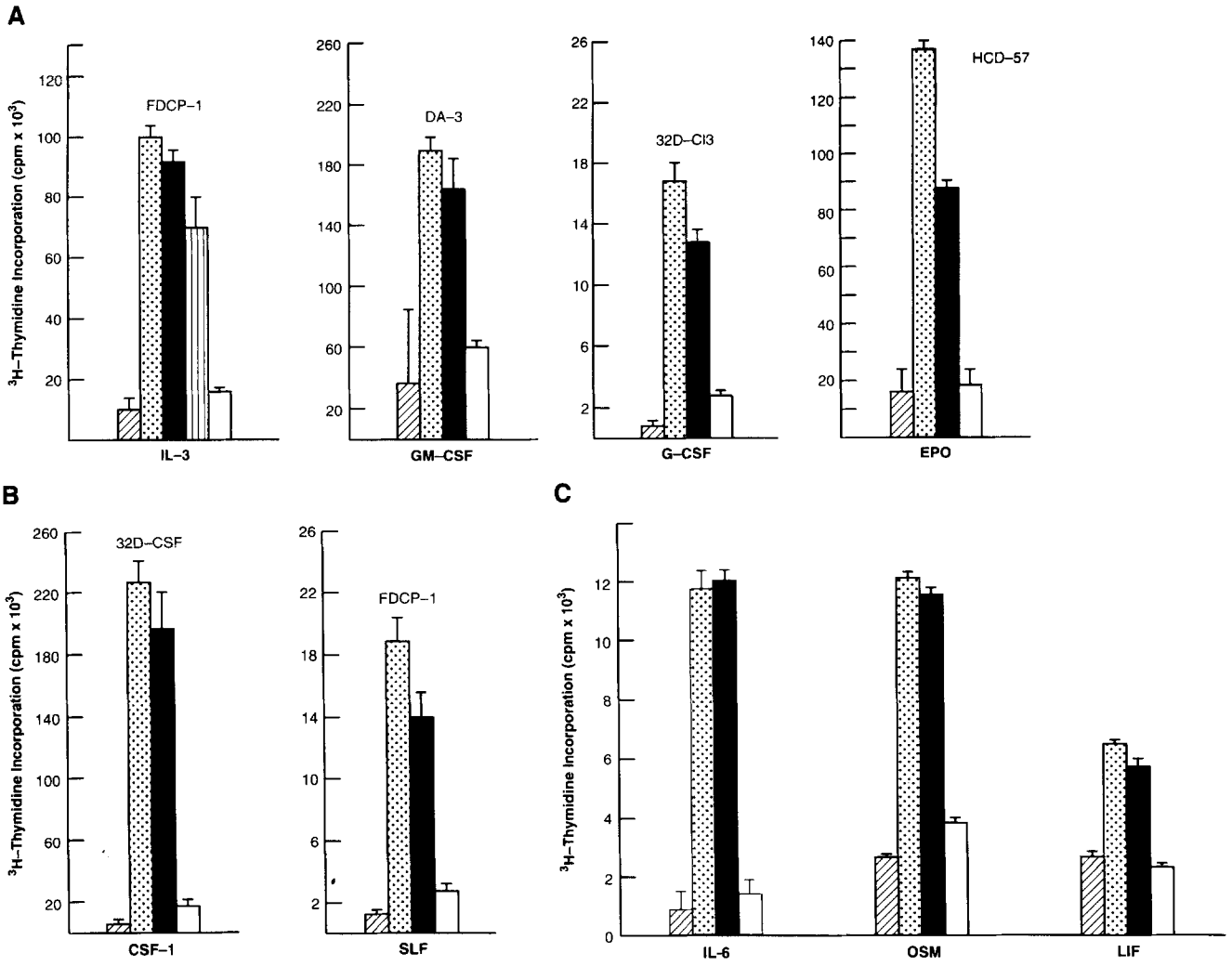

Figure 1. *c-raf* antisense oligonucleotides inhibit growth factor–induced proliferation of factor-dependent myeloid cell lines. Cell lines responsive to specific growth factors were treated with *c-raf* sense, antisense, or nonsense oligonucleotides and were assayed for [^{3}H]thymidine incorporation as described in Materials and Methods. Cells growing in media alone or in media plus growth factors were used as controls. (*A*) FDCP-1 cells stimulated with IL-3 (30 ng/ml), DA-3 cells stimulated with GM-CSF (30 ng/ml), 32D-Cl3 cells stimulated with G-CSF (50 ng/ml), and HCD-57 cells stimulated with EPO (5 U/ml); (*B*) 32D-CSF cells stimulated with CSF-1 (50 ng/ml) and FDCP-1 cells stimulated with SLF (50 ng/ml); (*C*) DA-1A cells stimulated with IL-6 (50 ng/ml), LIF (200 ng/ml), or OSM (200 ng/ml). Results are reported as the mean ± SD of triplicate wells from a single experiment. Experiments were repeated at least three to four times with similar results. ▨, media; ⊡, growth factor; ■, sense; ▥, nonsense; □, antisense.

we next evaluated the effect of *c-raf* oligonucleotides on proliferation of DA-1A cells stimulated with IL-6, LIF, or OSM. *c-raf* antisense oligonucleotides inhibited IL-6-, LIF-, and OSM-induced proliferation of DA-1A cells by >95% in contrast to sense-treated control oligonucleotides, which had little or no effect on proliferation (Fig. 1 *C*). *c-raf* antisense oligonucleotides also specifically inhibited IL-6–induced proliferation of B9 pro-B lymphoid cells by >95% (data not shown). Thus, Raf-1 is required to transduce the proliferative signal through different structural classes of hematopoietin receptors that are known to activate intracellular tyrosine kinases but that do not themselves contain intrinsic tyrosine kinase activity (4).

We next examined whether Raf-1 was required for proliferation of cells stimulated with CSF-1 or SLF, whose receptors are members of the tyrosine kinase receptor class and contain intrinsic tyrosine kinase activity (2). Similar to the effect of the oligonucleotides on cells stimulated with growth factors that activate the hematopoietin receptor class, *c-raf* antisense oligonucleotides also inhibited growth factor–induced proliferation of cells stimulated with CSF-1 or SLF by >95% in contrast to sense oligonucleotides, which showed no significant effect (Fig. 1 *B*). These results demonstrate that Raf-1 is also required for proliferation of cells stimulated with growth factors that transduce the growth factor–regulated signal through activation of intrinsic tyrosine kinases.

c-raf Antisense Oligonucleotides Inhibit Expression of the Raf-1 Protein. To determine whether *c-raf* antisense oligonucleotides were specifically inhibiting *c-raf* gene expression, we evaluated the effect of the oligonucleotides on Raf-1 protein expression in FDCP-1 cells stimulated with IL-3 by Western blot analysis (Fig. 2). Whole-cell lysates of untreated and sense

oligonucleotide– and antisense oligonucleotide–treated FDCP-1 cells stimulated with IL-3 were probed with anti–Raf-1 (SP63) antisera raised against synthetic peptides corresponding to COOH-terminal amino acid sequences of Raf-1 (31). The anti-Raf (SP63) antisera specifically detected a 74-kD Raf protein band that was competed by the peptide in untreated FDCP-1 control cells (Fig. 2) (31). c-*raf* antisense oligonucleotides completely inhibited Raf-1 protein expression after 36 h of treatment in contrast to sense oligonucleotides that had no effect on Raf-1 expression (Fig. 2). A higher molecular weight protein that was detected by the anti-Raf antisera and was not competed by the peptide in the oligonucleotide-treated cells was also observed in untreated control cells on longer exposures (>1 min) (Fig. 2). In contrast, neither sense nor antisense oligonucleotides effected expression of A-Raf, a closely related protein normally expressed in FDCP-1 cells (Fig. 2) (32), indicating that loss of c-*raf* gene expression was specifically related to the effect of the oligonucleotides and was not the result of an overall decrease in the expression of cellular proteins.

To demonstrate further that c-*raf* antisense-mediated inhibition of CSF-induced proliferation of factor-dependent cell lines was specifically related to loss of Raf-1 protein expression, we evaluated the effect of the oligonucleotides on IL-3–induced proliferation of an NFS-60 cell line infected with a retrovirus expressing the v-*raf* gene (35), which does not contain sequences recognized by the antisense oligonucleotides and therefore should not be affected (Fig. 3). c-*raf* antisense oligonucleotides inhibited IL-3–induced proliferation of uninfected NFS-60 cells by >95%; however, IL-3–induced proliferation of NFS-60 cells expressing the v-*raf* gene was inhibited by only 26% in comparison with sense-treated control cells, indicating that expression of the v-*raf* gene can overcome c-*raf* antisense inhibition of proliferation (Fig. 3). Taken together, these results demonstrate that c-*raf* antisense oligonucleotides inhibit the expression of the Raf-1 protein and that Raf-1 expression is specifically required for growth factor–induced proliferation.

The Raf-1 Kinase Is Activated by IL-4 and Is Required for IL-4–induced Proliferation of Factor-dependent Cell Lines. Since results of previous experiments had indicated that Raf-1 was not activated in cells stimulated with IL-4 (16), we evaluated the requirement for Raf-1 in IL-4–induced proliferation using the antisense approach. In contrast to the results of previous experiments (16), c-*raf* antisense oligonucleotides inhibited both IL-2– and IL-4–induced proliferation of CTLL-2 T cells by >95% (Fig. 4 *A*). However, IL-4 stimulation of CTLL-2 cells induced a fourfold lower proliferative response than IL-2 stimulation (Fig. 4 *A*), suggesting that the number of cells in the culture that responded to IL-4 may have been insufficient for detection of Raf-1 activation in the phosphorylation-induced shift and in vitro kinase assay. c-*raf* antisense oligonucleotides also inhibited IL-4–induced proliferation of 32D-Cl23 myeloid cells by 95% (data not shown) and IL-4–dependent FDCP-1 cells by 94% (Fig. 4 *B*).

Attempts to isolate an IL-4–dependent CTLL-2 T cell line from our cultures were unsuccessful; therefore, we evaluated IL-4–induced activation of Raf-1 kinase activity using the IL-4–dependent FDCP-1 cell line. IL-4 activation of Raf-1 was tested in kinase assays using either MBP or kinase-inactive GST-MEK$^-$ as a substrate (36) (Fig. 5, *A* and *C*). Raf-1 kinase activity was activated within 5 min, increased between 5 and 10 min, and declined by 30 min after the addition of IL-4 to growth factor–deprived FDCP-1 cells (Fig. 5, *A* and *C*). Concomitant with increased kinase activity, we also observed a shift in Raf-1 mobility, and the kinetics of mobility shift paralleled the increase and decrease in kinase activity (Fig. 5 *B*). The rapid and transient activation of Raf-1 in

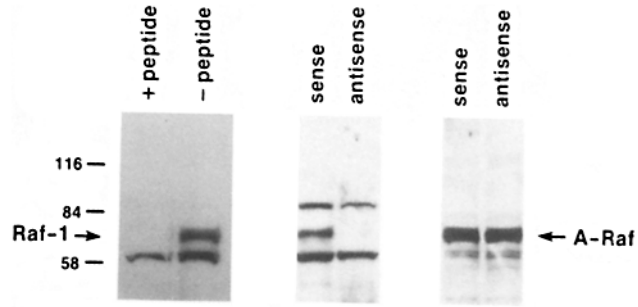

Figure 2. c-*raf* antisense oligonucleotides specifically inhibit Raf-1 protein expression. FDCP-1 cells treated with c-*raf* sense or antisense oligonucleotides as previously described were lysed 36 h after the addition of IL-3 (30 ng/ml), and whole protein lysates were analyzed for Raf-1 protein expression by Western blot. Untreated FDCP-1 cells stimulated with IL-3 were used for the media control. In media control lanes, 100 μg of protein from untreated cells was analyzed in the presence (+) or absence (–) of the competing peptide using anti-Raf (SP63) antisera, which detect the 74-kD Raf-1 protein band. In sense and antisense lanes, 45 μg of protein from oligonucleotide-treated cells was analyzed for expression of Raf-1 using SP63 antisera, and the 69-kD A-Raf protein band was detected by probing a parallel blot with anti–A-Raf antisera. A nonspecific higher molecular weight protein band that was detected by SP63 antisera in the oligonucleotide-treated cells was also observed in the media control lanes on longer exposures (>1 min).

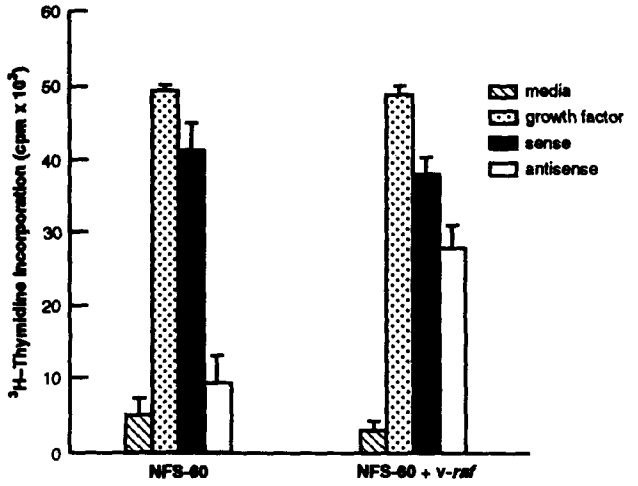

Figure 3. Expression of v-*raf* blocks c-*raf* antisense inhibition of IL-3–induced proliferation. NFS-60 cells that express the v-*raf* gene and the parental NFS-60 cell line were treated with c-*raf* oligonucleotides and evaluated for the effect of the oligonucleotides on IL-3–induced proliferation as described in Fig. 1.

cells stimulated with IL-4 indicates that Raf-1 is a component of IL-4–activated signal transduction pathways.

c-raf Antisense Oligonucleotides Inhibit CSF-induced Murine Bone Marrow Colony Formation. To investigate the requirement for Raf-1 in the growth of normal bone marrow cells, we next examined the effect of c-*raf* oligonucleotides on CSF-induced colony formation of murine progenitor cells. Unseparated and purified Lin$^-$ murine bone marrow–derived progenitor cells were treated with c-*raf* sense or antisense oligonucleotides and then plated in soft agar colony assays in the presence of IL-3, GM-CSF, or CSF-1. Cell viability was >90% for all samples, as assessed by trypan blue exclusion counting before plating. c-*raf* antisense oligonucleotides inhibited IL-3– and CSF-1–induced colony formation of unseparated bone marrow cells by 87 and 86%, respectively, and inhibited GM-CSF–induced colony formation by 65% (Table 1). Colonies growing in the presence of the antisense oligonucleotides were significantly smaller (<50 cells) than those formed by normal or sense-treated cells (>200 cells) but showed the same morphological distribution of colony types. Increasing the oligonucleotide concentration did not result in increased inhibition of GM-CSF–induced colony formation (data not shown). Lineage-negative bone marrow cells represent a population of cells that is enriched for primitive hematopoietic progenitors (~2.0% of unseparated bone marrow) (27), whereas unseparated bone marrow includes more committed progenitors. Therefore, to evaluate the requirement for Raf-1 in primitive progenitor cell growth, the effect of c-*raf* oligonucleotides on CSF-induced colony formation of Lin$^-$ cells was also evaluated. C-*raf* antisense oligonucleotides inhibited IL-3–, CSF-1–, and GM-CSF–induced colony formation of Lin$^-$-purified progenitors by >98% in comparison with sense oligonucleotides, which had little or no effect on colony formation (Table 1 and Fig. 6). Thus, Raf-1 is absolutely required for CSF-induced growth of progenitor-enriched bone marrow cells.

c-raf Antisense Oligonucleotides Inhibit CSF-induced Human Bone Marrow Colony Formation. The c-*raf* antisense oligonucleotides are complementary to the region surrounding the translation start site of both the murine and the human c-*raf* gene; therefore, the effect of c-*raf* oligonucleotides on growth factor–induced colony formation of unseparated and purified CD34$^+$ human bone marrow progenitors was evaluated using identical oligonucleotides. The CD34 cell surface antigen is expressed on immature hematopoietic precursors, and populations of CD34$^+$ cells are highly enriched for human hematopoietic stem cells (37, 38). c-*raf* antisense oligonucleotides inhibited GM-CSF–induced colony formation by 48% and IL-3 induced colony formation by 56% in unseparated human bone marrow cell populations (Table 2). However, similar to the results obtained with purified Lin$^-$ murine progenitors, c-*raf* antisense oligonucleotides completely inhibited colony formation (>95%) of CD34$^+$ purified progenitors stimulated with these same cytokines (Table 2). The few colonies formed in the presence of the antisense oligonucleotides were significantly smaller than colonies of untreated or sense-treated cells, and colony formation was not inhibited in a lineage-specific manner. Thus, similar to the result with murine bone marrow cells, Raf-1 was absolutely required for CSF-induced colony formation of primitive human progenitors cells.

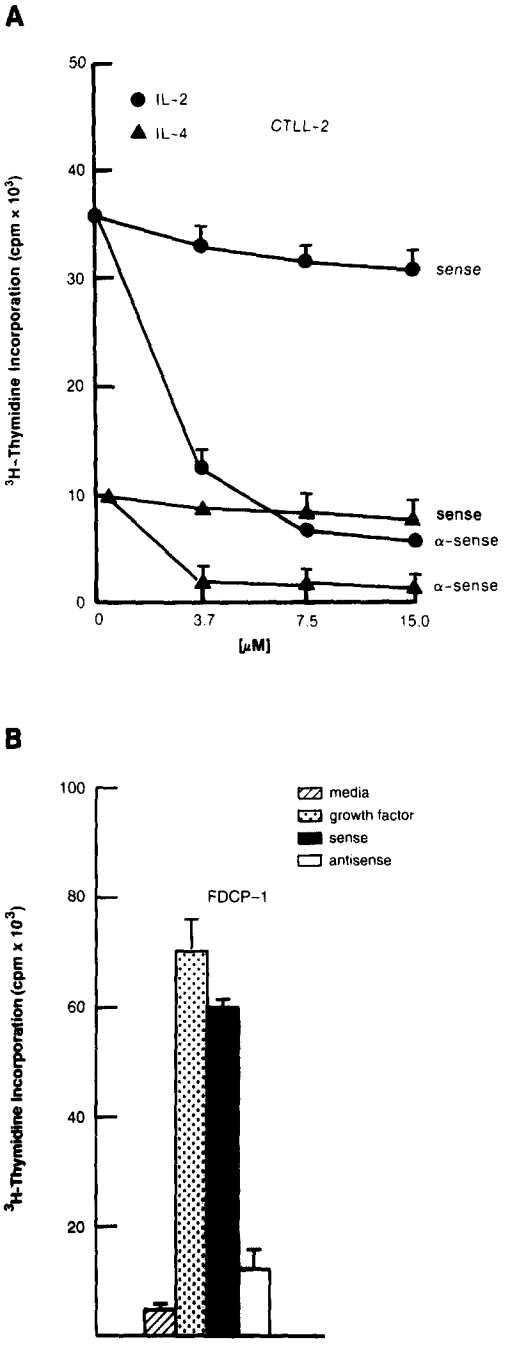

Figure 4. Raf-1 is required for IL-4–induced proliferation of CTLL-2 T cells and FDCP-1 myeloid cells. (*A*) CTLL-2 T cells treated with c-*raf* oligonucleotides at the concentrations indicated and stimulated with IL-2 (100 U/ml) or IL-4 (40 ng/ml). (*B*) IL-4–dependent FDCP-1 cells treated with c-*raf* oligonucleotides (7.5 μM) and stimulated with IL-4 (40 ng/ml). The effects of c-*raf* oligonucleotide treatment on IL-4– and IL-2–induced proliferation were evaluated by [^{3}H]thymidine incorporation.

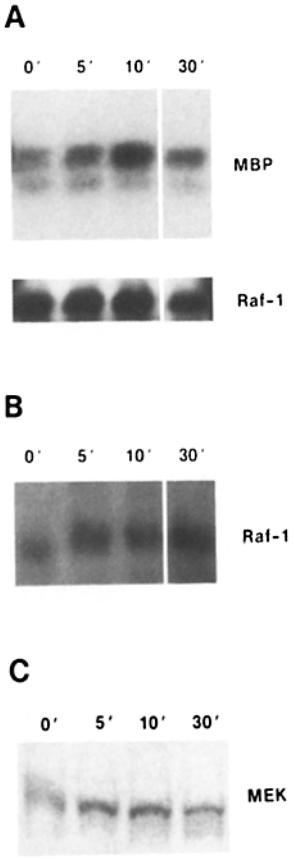

Figure 5. IL-4 activates Raf-1 kinase activity in IL-4–dependent FDCP-1 cells. (*A*) Raf-1 kinase activity in FDCP-1 cells stimulated with IL-4 as measured by Raf-1 phosphorylation of MBP. Phosphorylation of MBP was detected by autoradiography, and the filter was then probed with anti-Raf SP63 antisera to visualize the amount of Raf-1 present in the kinase reaction. (*B*) Raf-1 protein from the same immunoprecipitation was evaluated for IL-4–induced electrophoretic retardation. Immunoprecipitated proteins were resolved on a 7.5% SDS–polyacrylamide gel and analyzed by Western blot using SP63 antisera. The IL-4–induced shift in the molecular weight of Raf-1 cannot be detected on the 4–20% polyacrylamide gel used to resolve the MBP band. (*C*) Raf-1 kinase activity in IL-4–stimulated FDCP-1 cells was evaluated by phosphorylation of kinase-inactive GST-agarose affinity-purified MEK. Relative activity for each time point as determined by phosphorimager analysis was 29 (0 min), 42 (5 min), 50 (10 min), and 33 (30 min) $\times 10^3$ cpm. Equal amounts of Raf-1 protein were loaded for each sample (data not shown).

Discussion

To examine the functional requirement for Raf-1 for the growth of hematopoietic cells, we used c-*raf* antisense oligonucleotides to inhibit Raf-1 expression in growth factor–dependent myeloid cell lines and in normal hematopoietic progenitors. The antisense approach has been previously used in the functional analysis of numerous genes (39–41). In our experiments, c-*raf* antisense oligonucleotides inhibited growth factor–induced proliferation of leukemic cell lines stimulated with IL-2, IL-3, GM-CSF, EPO, G-CSF, CSF-1, and SLF. Thus, Raf-1 is required for growth factor–induced proliferation through stimulation of both the hematopoietin class of receptors and the tyrosine kinase receptor class. In addition, using the antisense approach, we have determined that Raf-1 is required for proliferation induced through the receptors for LIF, IL-6, and OSM, which represent a subclass of the hematopoietin receptor family that transduces growth factor–activated signals through association with the gp130 receptor subunit (7, 33, 34).

Results of previous experiments had indicated that one member of the hematopoietin receptor family, IL-4, did not activate the Raf-1 kinase in a T cell line (16). However, *c-raf* antisense oligonucleotides inhibited IL-4–induced proliferation in both myeloid and T cell lines, indicating that Raf-1 was required for IL-4–induced proliferation of these cells. The difference in these results may be explained by the fact that previous experiments evaluating IL-4–induced activation of Raf-1 were conducted with IL-2–dependent cells that prolifer-

Table 1. *Effect of c-raf Oligonucleotides on CSF-driven Murine Bone Marrow Colony Formation*

	Unseparated bone marrow* Number of colonies per plate§				Lin⁻ purified progenitors‡ Number of colonies per plate			
	Control	Sense	Antisense	Percent of Inhibition	Control	Sense	Antisense	Percent of Inhibition
CSF								
Media only	1 ± 0.8	–	–	–	0	–	–	–
IL-3	19.2 ± 0.8	20.3 ± 1.2	2.5 ± 0.8	87	45.0 ± 3.8	49.5 ± 4.4	0.5 ± 0.5	99
GM-CSF	67.7 ± 2.9	60.3 ± 2.5	21.3 ± 1.1	65	111.0 ± 4.0	112.2 ± 1.7	1.7 ± 1.1	98
CSF-1	59.5 ± 4.0	53.0 ± 3.8	7.8 ± 1.3	85	42.2 ± 2.5	56.2 ± 3.4	1.0 ± 0.8	98

* Normal bone marrow cells were aspirated from the femurs of BALB/c mice using complete RPMI 1640 containing 10% FCS and 3 mg/ml L-glutamine and penn/strep.

‡ Lin⁻ cells were selected using previously published techniques (19). Briefly, unseparated bone marrow cells were incubated with RB6-8S5, Mac-1 (Boehringer Mannheim), B220 (RB6-6B2), L3T4 (PharMingen), and Lyt-2 antibodies, which recognize myeloid- and lymphoid-specific cell surface antigens for 30 min at 4°C. Cells were then washed twice and incubated with anti-rat IgG–coated magnetic beads at a bead/cell ratio of 40:1 for 30 min at 4°C. Labeled cells were removed by a magnetic particle concentrator, and unlabeled cells (Lin⁻) were recovered in the supernatant. Unseparated bone marrow cells (3×10^5) or Lin⁻ cells (3×10^4) were treated with the oligonucleotides as described in Fig. 1 and then plated in triplicate in soft agar plates as previously described (19). Cultures were supplemented with IL-3 (30 ng/ml), GM-CSF (50 ng/ml), or CSF-1 (50 ng/ml) as indicated.

§ Colony formation was evaluated on day 7 for unseparated bone marrow cells and on day 12 for Lin⁻ cells. Results are the mean ± SEM for triplicate plates from two separate experiments. Percent inhibition was determined after adjusting for background.

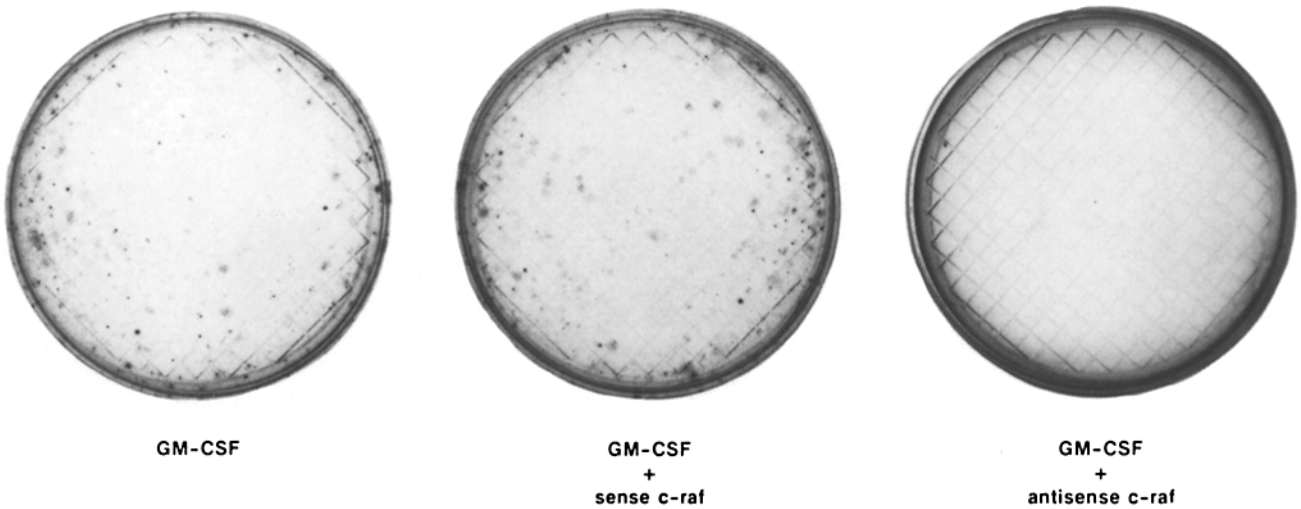

Figure 6. GM-CSF–induced colony formation of murine Lin⁻ bone marrow–derived progenitors is inhibited by *c-raf* antisense oligonucleotides. Purified Lin⁻ cells (3×10^4) either untreated or treated with *c-raf* antisense or sense oligonucleotides as described in Materials and Methods were stimulated with GM-CSF (30 ng/ml) and plated in soft agar. Day 7 colonies were stained with 0.1% INT for 3 d at 37°C and photographed.

ated in response to IL-4 but were not IL-4 dependent (16). Furthermore, our results indicated that the proliferative response to IL-4 in this cell line was submaximal when compared with the growth-promoting effects of IL-2, suggesting that the level of IL-4 receptor expression or the number of cells responding to IL-4 in the earlier biochemical experiments may have been insufficient to detect Raf-1 activation. Consistent with the results of the antisense experiments, our analysis of IL-4–induced activation of Raf-1 in an IL-4–dependent FDCP-1 cell line detected rapid and transient activation of Raf-1 kinase activity similar to that seen with other hematopoietic growth factors (42). It has been previously demonstrated that mitogen and growth factor–activated Raf-1 can phosphorylate nonphysiologic substrates such as MBP (36) and can phosphorylate and activate MEK, a recently identified physiologic substrate of Raf-1 (15, 43–45). The phosphorylation of MBP and MEK by Raf-1 protein immunoprecipitated from IL-4–stimulated cells demonstrates that IL-4 activates Raf-1 kinase activity. Numerous studies have established that the apparent increase in the molecular weight of Raf-1 that is observed in growth factor– or mitogen-stimulated cells is related to rapid phosphorylation of the Raf-1 protein (9, 16–20). A change in the electrophoretic mobility of Raf-1 in conjunction with activation of Raf-1 kinase activity in IL-

Table 2. *Effect of c-raf Oligonucleotides on CSF-induced Human Bone Marrow Colony Formation*

	Unseparated bone marrow* Number of colonies per plate§				CD34⁺ purified progenitors‡ Number of colonies per plate			
	Control	Sense	Antisense	Percent of Inhibition	Control	Sense	Antisense	Percent of Inhibition
CSF								
Media only	1.5 ± 0.5	—	—	—	8.5 ± 0.5	—	—	—
IL-3	72.5 ± 8.5	69.0 ± 7.0	32.0 ± 4.0	56	34.1 ± 0	28 ± 1.0	0.5 ± 0.5	99
GM-CSF	69.5 ± 1.5	50.5 ± 6.5	28.0 ± 7.0	48	21.0 ± 9.0	20 ± 2.5	7.0 ± 2.0	99

* Bone marrow cells obtained from human donors after informed consent were layered on lymphocyte separation medium to obtain light density cells. Cells were grown in IMDM supplemented with 25% FCS, 1% detoxified BSA penicillin (100 U/ml), streptomycin (100 μg/ml), and 3 mg/ml L-glutamine. Cells were stimulated with human IL-3 (30 ng/ml) or GM-CSF (50 ng/ml).

‡ CD34⁺ cells were obtained by positive selection using previously published techniques (15). Briefly, magnetic beads with the CD34-specific BL-3C5 mAb attached to them were added to cells at a bead/cell ratio of 1:1, and an anti-Fab antiserum (10 μl/10^7) was used for detachment of beads from positively selected cells. CD34⁺ cells were then recovered by magnetically separating the beads using a magnetic particle concentrator. Unseparated bone marrow cells (3×10^5) and CD34⁺ cells (3×10^4) were treated with oligonucleotides and evaluated in soft agar colony assays as described in Table 1.

§ Colony formation of both unseparated and human bone marrow cells was evaluated on day 12. Results are the mean ± SEM for triplicate plates from two separate experiments. Percent of inhibition was determined after adjusting for background.

4–stimulated cells suggests that, similar to the mechanism of Raf-1 activation seen with other growth factors, IL-4 also induces a rapid and transient phosphorylation of the Raf-1 protein. Additional experiments are required to determine the essential phosphorylation sites for IL-4–induced activation of Raf-1. Taken together with the results of previous experiments, the studies evaluating IL-4 activation of Raf-1 kinase activity indicate that the Raf-1 kinase is activated by all hematopoietic growth factors that have been tested to date.

The c-*raf* antisense oligonucleotides used in this experiment specifically inhibit expression of the Raf-1 protein without affecting expression of A-Raf, a closely related protein that is not homologous to Raf-1 in the region recognized by the antisense oligonucleotides. Similarities in the mechanism of activation and the downstream events regulated by *raf* family genes have suggested that A-Raf may substitute for Raf-1 in growth factor–regulated signaling pathways (46–48). However, since A-Raf but not Raf-1 was expressed in c-*raf* antisense–inhibited FDCP-1 cells, this result indicates that A-Raf does not substitute for the function of Raf-1 in IL-3–induced proliferation. The loss of Raf-1 expression in the absence of any effect on expression of a closely related protein demonstrated that loss of c-*raf* gene expression is specifically related to the effect of the oligonucleotides and is not related to an overall decrease in cellular protein levels. It did not, however, rule out the possibility that the oligonucleotides also affected expression of unrelated cellular proteins that were also required for growth factor–induced proliferation. Therefore, we further established the specificity of c-*raf* antisense oligonucleotide inhibition of growth factor–induced proliferation using an NFS-60 cell line containing a v-*raf* gene, whose expression is not affected by the antisense oligonucleotides. Expression of the v-*raf* gene significantly reversed c-*raf* antisense inhibition of IL-3–induced proliferation of NFS-60 cells, demonstrating that Raf-1 expression is specifically required for growth factor–induced proliferation. Since the NFS-60 cells expressing v-*raf* were not a clonal population of cells, partial inhibition of IL-3–induced proliferation of these cells may result from c-*raf* antisense inhibition of cells in the culture that have little or no expression of the v-*raf* gene.

Similar to the requirement for Raf-1 in growth factor–induced proliferation of progenitor cell lines, Raf-1 was absolutely required for colony formation of purified normal murine (Lin^-) and human ($CD34^+$) bone marrow–derived progenitor cells stimulated with IL-3, GM-CSF, or CSF-1. In addition, colony formation of unseparated murine bone marrow cells stimulated with IL-3 or CSF-1 was inhibited by 87 and 85%, respectively; however, GM-CSF–induced colony formation was inhibited only by 62%. Partial inhibition of GM-CSF–induced colony formation of unseparated murine bone marrow cells suggested that Raf-1 may not be required for the GM-CSF–induced growth of some normal progenitors. However, the morphological distribution of colony types normally induced by GM-CSF was not altered in c-*raf* antisense–treated plates, indicating that there was not a differential requirement for Raf-1 in different cell types. Alternatively, it was possible that the oligonucleotide concentration we used was insufficient to inhibit GM-CSF–induced colony formation completely. However, this does not seem likely, since increasing the oligonucleotide concentration did not result in increased c-*raf* antisense inhibition of GM-CSF–induced colony formation. Complete inhibition (>99%) of GM-CSF–induced colony formation of Lin^- progenitors demonstrates that Raf-1 is absolutely required for colony formation of progenitor-enriched bone marrow cells. However, our data do not exclude the possibility that the small GM-CSF–induced colonies in unseparated bone marrow cells that proliferate in the presence of c-*raf* antisense oligonucleotides arise from more mature progenitors that have a reduced requirement for Raf-1–mediated proliferative signals.

In comparison with their effects on colony formation of unseparated murine bone marrow cells, c-*raf* antisense oligonucleotides partially inhibited both IL-3– and GM-CSF–induced colony formation of unseparated human bone marrow cells. Differences in the effects of the oligonucleotides on murine versus human bone marrow cells are consistent with known structural differences between the IL-3 and GM-CSF receptor systems in human and mouse. Specifically, in humans, the IL-3 and GM-CSF receptors share a common β subunit that is the signaling component of these receptors, whereas in the mouse IL-3 can use either a common β subunit or a distinct β subunit (49–51).

Previous studies have suggested a role for Raf-1 in lineage determination in v-*raf*–transformed hematopoietic progenitor cells (52). However, the requirement for Raf-1 in the differentiation of normal bone marrow–derived progenitors could not be determined in the colony assays, since proliferation is required for differentiation of hematopoietic cells. Experiments designed to uncouple differentiation and proliferation are currently underway to determine whether Raf-1 is specifically required for differentiation of hematopoietic cells.

We have established that Raf-1 is required for growth factor–induced proliferation of leukemic murine progenitor cell lines and colony formation of normal murine and human bone marrow–derived progenitor cells regardless of the growth factor used to stimulate cell growth. A requirement for Raf-1 was established for growth factor–induced proliferation through activation of receptors that are members of the hematopoietin receptor family, including the IL-4 receptor and the receptors for IL-6, LIF, and OSM, which use the gp130 signaling molecule. Raf-1 is also required for proliferation induced through activation of the tyrosine kinase receptor class by CSF-1 and SLF. These results indicate that Raf-1 is a component of multiple signal transduction pathways that regulate hematopoietic cell proliferation and differentiation, and they demonstrate that activation of Raf-1 represents a common coupling mechanism for several different structural classes of hematopoietic receptors.

We thank J. Pierce (Laboratory of Cellular Metabolism and Biochemistry, National Cancer Institute [NCI]) for the gift of 32D-CSF cells, S. Ruscetti (Laboratory of Molecular Oncology, NCI) for HCD-57 cells, M. Schwabe (Laboratory of Biochemical Physiology, Biological Response Modifiers Program, NCI) for B9 cells, and P. Donovan (ABL Basic Research Program, NCI) for DA-1A cells. We also thank Maria Ortiz for assistance with the colony assays, K. Boudreaux, K. Mood, and J. Mikovits for oligonucleotide synthesis, and Dr. Dan Longo for review of the manuscript.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U. S. government.

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Received for publication 9 December 1994 and in revised form 5 January 1995.

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