Raf-1 Is a Necessary Component of the Mitogenic Response of the Human Megakaryoblastic Leukemia Cell Line MO7 to Human Stem Cell Factor, Granulocyte-Macrophage Colony-stimulating Factor, Interleukin 3, and Interleukin 9

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Abstract
We have examined the role of Raf-1 in the mitogenic response of the factor-deprived human megakaryoblastic leukemia cell line MO7 to recombinant human granulocyte-macrophage colony-stimulating factor, interleukin 3, interleukin 9, and stem cell factor by using c-raf antisense oligodeoxyribonucleotides. Uptake of oligodeoxyribonucleotides by MO7 cells was maximal at 5–10 h in culture, and oligomers remained stable in these cells for at least 24 h. Treatment of MO7 cells with the antisense oligomer resulted in intracellular oligomer/mRNA duplex formation followed by efficient translation blockade of c-raf-1. In contrast, sense and non-sense oligodeoxyribonucleotides failed to form intracellular duplexes and did not interfere with translation of c-raf-1, suggesting specific elimination of c-raf-1 by the antisense oligodeoxyribonucleotide. Furthermore, exposure of MO7 cells to c-raf-1 antisense prevented factor-induced nuclear translocation of Raf-1. Most importantly, proliferation of MO7 cells ([3H]thymidine incorporation) enabled by these growth factors was significantly reduced when the c-raf-1 antisense oligodeoxyribonucleotide was added to cultures, whereas the mitogenic response to these factors remained almost unaffected in the presence of sense and non-sense oligodeoxyribonucleotides.

Introduction
The product of the c-raf-1 protooncogene, Raf-1, is known to encode a 74 kilodalton cytoplasmic serine/threonine kinase. Various growth factors, including epidermal growth factor, nerve growth factor, acidic fibroblast growth factor, platelet-derived growth factor, and insulin as well as several hematopoietins are known to activate Raf-1 kinase and induce Raf-1 phosphorylation. It has, for example, been shown that GM-CSF and IL-3-directed growth of MO7 megakaryoblastic leukemia cells is associated with increased phosphorylation of Raf-1 (1). The kinases responsible for Raf-1 phosphorylation are presently not completely understood. There is, however, evidence to suggest that Ras-GTP is the major phosphorylation origin of Raf-1 (2, 3), and that the mitogen-activated protein kinase-kinase may act as a substrate for Raf-1 (4). Raf-1 phosphorylation may involve both serine and tyrosine residues, as has been reported for IL-2-mediated Raf-1 phosphorylation (5), but may also occur predominantly or exclusively on serine residues, as shown for signals delivered by GM-CSF, IL-3, macrophage-CSF, platelet-derived growth factor, epidermal growth factor, and insulin (1, 2, 6–10). Several lines of evidence suggest that Raf-1 couples signals triggered by many different kinds of mitogens, including growth factors for receptor tyrosine kinases, agonists for protein kinase C, G-protein agonists, and other nonreceptor kinases, to proliferative responses of various cell types (1–11).

Moreover, the viral homologue v-raf has been shown to cooperate with c-myc in abrogating factor dependence of murine FDC-P1 cells (12) and also in inducing leukemias and lymphomas (13). Elimination of Raf-1 may thus favor growth arrest, at least when the growth-promoting event is generated upstream of c-raf-1. In this regard, previous studies are of note, demonstrating that phorbol 12-myristate 13-acetate-mediated proliferation of NIH3T3 cells is relieved upon transfection of an antisense c-raf expression vector (14). Similarly, growth of NIH/3T3 cells can be blocked upon injection of an anti-ras-specific monoclonal antibody, and this growth arrest can be abrogated by introduction of v-raf (15).

Work demonstrating activation of Raf-1 by specific growth factors does not clearly settle the question of whether Raf is necessary or suitable for transduction of a growth factor signal, whereas inhibition of Raf does directly address this issue. In this study, an octadecamer oligodeoxyribonucleotide corresponding to codons 1–6 of human c-raf-1 was used to investigate its effect on growth promotion of myeloid leukemia cells directed by different growth factors. In these experiments, the megakaryoblastic human leukemia cell line

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1 The abbreviations used are: GM, granulocyte-macrophage; CSF, colony-stimulating factor; IL-3, interleukin 3 (other interleukins are designated similarly); rh, recombinant human; SCF, stem cell factor; AS, antisense; NS, nonsense; S, sense; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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MO7, known to respond proliferatively to rh GM-CSF, IL-3, IL-9, and SCF (16-18), served as a working model system.

Results and Discussion

Previous studies demonstrating growth factor-induced phosphorylation and activation of Raf-1 prompted investigations on the effect of c-raf-1 antisense oligodeoxyribonucleotides on [3H]thymidine uptake by MO7 cells, a human megakaryoblastic leukemia cell line known to respond proliferatively to various hematopoietic growth factors, including GM-CSF, IL-3, IL-9, and SCF. Antisense oligodeoxyribonucleotides efficiently enter cells and interfere with the expression of their cognate gene product. Most antisense oligodeoxyribonucleotides destabilize their target mRNA, making it susceptible to rapid degradation, and therefore enable studies on the biological role of their respective proteins (19-21).

We have used an octadecamer antisense oligodeoxyri bonucleotide complementary to the first six codons of human c-raf-1. c-raf specificity was confirmed by a computer-assisted GenBank screen. In pilot experiments (data not shown), the stability of oligonucleotides in cell culture medium was determined to be at least 36 h. To examine oligodeoxyribonucleotide uptake by MO7 cells, the MO7 line was cultured in the presence of radioactively labeled AS, S, or NS oligodeoxyribonucleotides for up to 24 h. Cells were washed several times, and radioactivity retained by the washed cell pellet was compared to the radioactivity left in the culture medium. Approximately 1% of the radioactivity was associated with the cell pellet within 1 h of culture. This fraction further increased to 5.9% of the total radioactivity within 5 h of culture and remained at that level for up to 24 h (Fig. 1). In order to assess oligodeoxyribonucleotide stability, denaturing gel electrophoresis was performed with lyophilized aliquots of lysed cells. In the intact cell pellets, AS, S, and NS oligodeoxyribonucleotides were stable for up to 24 h (Fig. 2).

Next, we assessed intracellular duplex formation of c-raf-1 mRNA and the respective oligodeoxyribonucleotides, as previously described by Holt et al. (22). To this end, MO7 cells were cultured in the presence or absence of the respective S or AS oligodeoxyribonucleotides for 4 h, followed by mRNA extraction. A S1 nuclease-resistant duplex of endogenous mRNA and the 5'-end-labeled oligomer were detectable in RNA extracted from MO7 cells when cultured for 4 h in the presence of AS oligomers, but not when cultured with S or NS oligomers (Fig. 3). We also failed to detect duplex formation by S1 nuclease assay when labeled oligomers were added after the culture period and immediately prior to RNA extraction, indicating that oligomer/mRNA duplexes had been formed intracellularly and not during RNA preparation (for details, see “Materials and Methods”).

The effect of the c-raf-1 antisense on DNA synthesis of MO7 cells directed by GM-CSF, IL-3, IL-9, SCF, and combinations thereof was investigated to more accurately explore the role of Raf-1 in growth stimulation and signaling of these hematopoietins. MO7 cells, which were maintained in 10% fetal calf serum and 2.5 ng/ml rh GM-CSF + 1 ng/ml rh IL-3, were first factor and serum deprived. As shown in Fig. 4, addition of AS c-raf-1, but not of S and NS oligomers, specifically inhibited factor-induced [3H]thymidine incorporation by MO7 cells. In these experiments, oligomers were used at a 10 μM concentration because pilot experiments using rh SCF as a stimulus had demonstrated optimum inhibition of MO7 growth by this dose (Fig. 5A). Furthermore, this dose was also shown to be effective in other systems (10, 21, 23). Viability of MO7 cells, determined by trypan blue exclusion, was >80% for oligomer-treated as well as untreated cultures (data not shown), thus ruling out that the antisense effect was cytotoxic. Addition of a 10-fold excess of a second complementary sense oligomer to c-raf-1 completely reversed the antisense oligomer effects by hybridization competition (Fig. 5B). Furthermore, addition of 10 μM antisense to c-raf-1 also almost completely decreased intracellular Raf-1 levels in MO7 cells (Fig. 6). Reduction of Raf-1 was specific in that levels of other proteins probed on the same blot did not change (data not shown). Immunofluorescence using an anti-Raf antibody confirmed these results on the morphological level by demonstrating that Raf-1 kinase is dispersed throughout the cell in quiescent, serum-

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starved, and factor-deprived MO7 cells, but rapidly moved into the nucleus after factor stimulation. This nuclear translocation has already been shown by others to be the consequence of factor-induced phosphorylation and activation of Raf-1 in NIH/3T3 fibroblasts (24). As shown in Fig. 7, antisense c-raf-1 oligomers highly reduced (but not completely abolished) the intranuclear levels of Raf-1.

In summary, growth of MO7 cells stimulated by GM-CSF, IL-3, IL-9, SCF, and combinations thereof can be specifically inhibited by an antisense oligomer to c-raf-1, leading to a substantial decrease of intracellular Raf-1 levels. These data, when taken together with the observed growth factor-directed activation of Raf-1 (1), show that Raf-1 is a crucial component of the mitogenic signal delivered by GM-CSF, IL-3, IL-9, and SCF.

Materials and Methods
Reagents. Highly purified rh GM-CSF and rh IL-3 were kindly provided by Dr. S. Gillis, Immunex Corp. (Seattle, WA). Recombinant human IL-9 was a gift of Drs. S. Clark and G. Wong, Genetics Institute (Cambridge, MA). Recombinant human SCF was kindly provided by Dr. Ch. Zsebo, Amgen (Thousand Oaks, CA).

Cell Lines. The human GM-CSF- and IL-3-dependent cell line MO7 was obtained from Dr. H. G. Drexler, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and was originally derived from the peripheral blood of an infant with acute megakaryoblastic leukemia (15). The cell line was cultured in standard culture medium consisting of RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. MO7 cells were maintained in culture in the presence of rh GM-CSF (2.5 ng/ml) and rh IL-3 (1.0 ng/ml).

Preparation of Oligodeoxyribonucleotides, Oligodeoxynucleotide Design, Stability Assay, and Determination of Intracellular Duplex Formation. Phosphothionated-modified antisense (5'-TCCCTGTATGTTGCCTCAT-3') and sense (5'-ATGGAGCCATAACAGGGA-3') oligodeoxyribonucleotides corresponding to codons 1–6 of human c-raf-1 or a randomly generated non-sense oligomer with an overall base composition identical to that of the antisense oligomer (5'-TTTTTGACACCAGCTGCC-3') were prepared using a Milligen 7500 synthesizer and purified by reverse phase HPLC. Oligodeoxyribonucleotide uptake studies were performed essentially as previously described (25). Briefly, oligomers were 5'-end labeled with [γ-32P]ATP with the bac-
teriophage T4 polynucleotide kinase and purified by denaturing polyacrylamide gel electrophoresis. Approximately $5 \times 10^5$ cpm of 5'-labeled oligodeoxynucleotides were added to $4 \times 10^6$ MO7 cells in culture medium. Cells were incubated at 37°C in 7% CO$_2$ in air for up to 24 h, and aliquots were collected at various time points as indicated. Cells were pelleted, and the supernatant was harvested and saved. Cells were then washed twice in PBS (GIBCO) and pelleted, and the cell pellet was lysed in 0.1 ml TBS (10 mM Tris-HCl, pH 7.4–150 mM NaCl-1% NADSO$_4$), followed by phenol extraction. Aliquots of the aqueous phase, the cell wash, and culture medium supernatants were analyzed by liquid scintillation counting. The percentage of oligodeoxynucleotides taken up by the cells was calculated by dividing the counts of the aqueous phase by the total counts (aqueous phase plus cell wash plus culture medium supernatant). To examine stability of oligodeoxynucleotides, aliquots of the aqueous phase were lyophilized and redissolved in 20 ml of loading buffer (80% deionized formamide, 0.01% bromphenol blue, and 0.01% xylene cyanole FF), followed by electrophoresis through a 12% denaturing polyacrylamide gel.

Experiments designed to analyze intracellular duplex formation were performed as described by Holt et al. (22). Briefly, oligodeoxynucleotides were 5'-end labeled as described above to a specific activity of $2 \times 10^7$ to $4 \times 10^7$ cpm/mg and added to MO7 cells in culture medium at a concentration of 100 ng/ml. After 4 h, following several washing steps in prewarmed PBS, cells were lysed in 100 ml of lysis buffer (0.25 M Tris-HCl, pH 7.4–10 mM NaCl-3 mM MgCl$_2$-0.05% Nonidet P-40-0.5% sodium dodecyl sulfate-100 mg Proteinase K/ml). As a carrier, a 10,000-fold excess of an unlabeled oligomer was added. Following phenol-chloroform extraction and ethanol precipitation, a single nucleic acid protection assay was performed as previously described (26). Products were analyzed on a 15% denaturing polyacrylamide gel. In order to confirm that duplexes had been formed intracellularly, a control experiment was performed in which an equal amount of cell-associated radioactivity together with an excess of unlabeled carrier oligomer was added to lysates of cells that had been cultured in the absence of labeled oligonucleotides ("add back control").

Aliquots of purified oligomers dissolved in ammoniated water were evaporated in a speed vac concentrator and resuspended in RPMI 1640 prior to use.

$[^3H]$Thymidine Incorporation Assay. Growth factor- and serum-deprived MO7 cells were first cultured in the presence or absence (w/o) of c-raf-1 antisense (α-sense) or sense oligodeoxynucleotide (72 h) before growth factors were added for a further 18 h. Raf-1 protein was detected by Western blotting using Raf-1-specific monoclonal antibody SP63. Lane 1, treatment of MO7 cells with medium only; Lane 2, with IL-3 (50 ng/ml); Lane 3, with SCF (100 ng/ml); Lane 4, with IL-9 (50 ng/ml); Lane 5, with GM-CSF (50 ng/ml); Lane 6, with a combination of all factors.

**Fig. 6.** c-raf-1 antisense decreases intracellular Raf-1 levels in MO7 cells. Growth factor- and serum-deprived MO7 cells were first cultured in the presence or absence (w/o) of c-raf-1 antisense (α-sense) or sense oligodeoxynucleotide (72 h) before growth factors were added for a further 18 h. Raf-1 protein was detected by Western blotting using Raf-1-specific monoclonal antibody SP63. Lane 1, treatment of MO7 cells with medium only; Lane 2, with IL-3 (50 ng/ml); Lane 3, with SCF (100 ng/ml); Lane 4, with IL-9 (50 ng/ml); Lane 5, with GM-CSF (50 ng/ml); Lane 6, with a combination of all factors.
Fig. 7. Immunofluorescence of M07 cells with anti-rat antibody SP63. Before cells were stimulated with recombinant human stem cell factor, an incubation was performed in the presence (A) or absence (B, medium only) of c-raf-1 antisense oligomers. Upon stimulation with SCF, fluorescence translocates into the perinuclear and nuclear space (B). Incubation with antisense c-raf-1 prior to SCF stimulation significantly reduces staining and prevents translocation of fluorescence (A). In experiments in which cells were stimulated with rh GM-CSF, rh IL-3, rh IL-9, comparable results were obtained. No prevention of translocation of fluorescence was noted when cells had been preincubated with a c-raf sense oligomer (not shown).

factors, including GM-CSF (50 ng/ml), rh IL-3 (50 ng/ml), rh SCF (100 ng/ml), rh IL-9 (50 ng/ml), or various combinations thereof. Following an additional 18-h incubation period, cells were pulsed with [H]thymidine (1 μCi/well) for 6 h prior to harvesting using a PHD cell harvester (PHD, Cambridge, MA). In selected experiments, a 10-fold excess of complementary sense oligomers was added to cultures along with 10 μM antisense oligomer. [H]thymidine incorporation was determined by scintillation counting. Control cultures were performed in the absence of growth factors or added oligomers.

Detection of RAF-1 Protein. M07 cells, cultured under serum- and growth-factor-deprived conditions, as described above, were lysed in sodium dodecyl sulfate sample buffer (9). Lysates (equivalents of 2.5 × 10^6 cells/lane) were then loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. Equal loading was confirmed by staining total proteins on a parallel blot with Ponceau S (Sigma, Munich, Germany). RAF-1 was probed by Western blotting using a RAF-1 peptide antiserum (anti-SP63; batch no. 72255), as described in detail elsewhere (9).

Immunofluorescence. Cytospin preparations of cells exposed to rh GM-CSF, rh IL-3, rh SCF, or rh IL-9, or control cells (medium treatment only) were washed in PBS and fixed in 3.7% formaldehyde for 10 min. Cells were blocked for 30 min in TBS, 0.25% Triton, and 5% normal goat serum followed by a 1-h incubation with anti- SP63 antibody (1:1000 dilution) at 4°C. The slides were then washed with TBS and 0.25% Triton and incubated with a secondary antibody (rhodamine-conjugated anti-rabbit; Sigma). After the final washing step in TBS and 0.25% Triton, cells were fixed with 3.7% formaldehyde, and slides were photographed with a Zeiss microscope at 1000×.

References
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