Expression Cloning of a cDNA Encoding a Novel Human Hematopoietic Growth Factor: Human Homologue of Murine T-Cell Growth Factor P40

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We used functional expression cloning in mammalian cells to identify a cDNA clone encoding a hematopoietic growth factor that is mitogenic for the factor-dependent human megakaryoblastic leukemic cell line, M07E. Analysis of the sequence of this cDNA revealed striking similarity to that of a recently reported novel murine growth factor for helper T-cell clones designated T-cell growth factor P40. The mRNA for the human P40 protein is expressed by several different human T-cell lines and by mitogen-stimulated peripheral blood lymphocytes. The recombinant protein displays substantial size heterogeneity similar to that of other glycoprotein cytokines. These properties plus the observation that this cytokine may well act within both the lymphoid and myeloid lineages warrant the designation of P40 as interleukin-9.

REGULATION OF the growth and differentiation of cells within the hematopoietic system has provided a useful model for analysis of growth factor regulation in cellular development. In the human system, much of what is known about the hematopoietic growth factors derives from analysis of growth of normal progenitor cells in culture. However, recent development of several human factor-dependent leukemic1,2 cell lines has provided some new tools for further characterization of the regulators of hematopoiesis. We extensively analyzed the growth factor responsiveness of one such line, designated M07E, which was originally derived from an infant with acute megakaryoblastic leukemia.3 M07E cells are highly responsive to granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3). Analysis of the conditioned medium from a human T-cell leukemia virus (HTLV-I)-transformed T-cell line, C5M2, indicated that these cells elaborate an M07E-growth-promoting activity distinguishable from the known human cytokines. We used functional expression cloning in monkey COS-1 cells to identify a cDNA encoding this novel growth-promoting activity. The novel human growth factor thus identified displays striking similarity to the newly identified murine T-cell growth factor, P40.2 Our finding that the human homologue of a murine T-cell growth factor is mitogenic for a human megakaryoblastic leukemic cell line suggests that this molecule, like many of the interleukins, may serve multiple functions in regulating growth and development of cells in different lineages.

MATERIALS AND METHODS

Cell lines and cell culture. C5M2 and C10MJ2 cells were cultured in RPMI 1640 containing 20% heat-inactivated fetal calf serum (HI-FCS). UCD-144-MLA2 cells were grown in RPMI 1640 plus 10% HI-FCS, and Mo5 cells were grown in Iscove’s medium plus 20% HI-FCS. M07E cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 20% HI-FCS and 10 U/mL bacterial human IL-3 (provided by the Genetics Institute Development Laboratory). COS-1 cells were routinely maintained in Dulbecco’s modified Eagle’s medium with 10% HI-FCS. Human peripheral blood lymphocytes were pooled from several different donors and resuspended in RPMI 1640 with 5% HI-FCS. UCD-144-MLA cells were induced with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and C5M2, C10MJ2, Mo, and peripheral blood lymphocytes were induced with 0.1% phytohemagglutinin (PHA) plus 5 ng/mL PMA for 24 hours for RNA isolation and 48 hours for bioassay.

Bioassay. The M07E cell proliferation assay was performed as follows: The cells were subcultured at 3 x 105 cells/mL 2 to 4 days before use in the assay. On the day of assay, the cells were collected, washed, and 0.1 mL containing 10,000 cells was plated in each of the wells of a microtiter plate; 0.1 mL of dilutions of samples, either alone or mixed with dilutions of antibodies, was added to the wells. The cells were allowed to grow at 37°C for 72 hours, after which each well was pulsed for 4 hours with 0.5 µCi [3H]-thymidine (New England Nuclear, Boston, MA). Finally, the cells were harvested, and radioactivity was determined by liquid scintillation counting.

The neutralizing antibody to human GM-CSF was prepared by immunizing sheep with recombinant human GM-CSF,5 and the neutralizing antibody to human IL-6 was prepared by immunizing a rabbit with recombinant.6

Isolation and characterization of the pC5.22-3 cDNA clone. A cDNA library containing 250,000 clones was prepared beginning with 5 µg mRNA isolated from PMA/PHA-induced C5M2 cells with the expression vector pX M as described previously.10 DNA pools of 250 to 500 clones were individually transfected into COS-1 cells using the diethylaminoethyl-Dextran protocol with chloroquine treatment as described previously.10 The conditioned medium, harvested after 72 hours, was tested in the M07E bioassay at final concentrations of 10% and 2%. The one positive pool was subdivided using sib-selection, ultimately yielding a single clone designated pC5.22-3. The insert of this cDNA was sequenced by the dideoxy chain-termination method11 on supercoiled templates with synthetic oligonucleotide primers.12

RNA and protein analysis. RNA blot analysis was performed as described previously10 with 5 µg of each mRNA sample and a 1.2% agarose gel. The transfected COS-1 cells were pulse-labeled with 0.5 µCi [35S]-methionine in 0.5 mL methionine-free Dulbecco’s modified Eagle’s medium for 4 hours, 72 hours after transfection as described previously.16 Ten-microtiter samples were analyzed by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis with the Laemmli buffer system and a 12% gel as described previously.17 The reference protein sample was purchased from Bethesda Research Laboratories (Gaithersburg, MD).

RESULTS

Identification of a cDNA encoding a novel M07E growth-promoting activity. Our analysis of the growth responsiveness of leukemic cell lines to conditioned medium prepared from different cell sources revealed that C5M2 cells elabo-
rate a combination of cytokines that is particularly mitogenic for M07E cells (Fig 1A). Much of this mitogenic activity could be eliminated by preincubation with neutralizing antisera to GM-CSF and IL-6, factors known to be produced by C5M2J cells. However, a combination of RNA blot and bioassay analysis (data not shown) indicated that the antibody-resistant mitogenic activity could not be accounted for by IL-1, IL-3, IL-4, or lymphotoxin, other cytokines that support thymidine uptake by M07E cells. Although the residual stimulation was modest relative to that observed with either GM-CSF or IL-3, it was typically more than 10-fold higher than that observed without growth factors added. These results provided the basis for a sensitive bioassay suitable for use in expression cloning.

To identify a cDNA clone encoding the M07E growth-promoting activity, we prepared a cDNA expression library from mRNA isolated from C5M2J cells 24 hours after exposure to PHA and PMA, a treatment that enhances the

Fig 1. Growth response of M07E cells to medium conditioned by C5M2J cells and to pC5.22-3-transfected COS-1 cells. The M07E growth factor activities of conditioned medium from induced C5M2J cell line (A) and pC5.22-3-transfected COS-1 cells (B) were tested without (---O---; ---X---) or with (---Δ---) antibodies to human GM-CSF and human IL-6 as described in the Materials and Methods section. Background (---●---) of the assay is as indicated.
level of the novel factor. We screened 550 pools from the library, each containing 250 to 500 clones, for the ability to direct expression of M07E growth-promoting activity when transfected into COS-1 cells. Only one pool of clones yielded conditioned medium with significant activity when tested with the antisera against GM-CSF and IL-6. This pool was further subdivided until an individual plasmid, designated pC5.22-3, was identified which by itself directed high levels of expression of the novel activity (Fig 1B). Pulse-labeling experiments of the transfected cells confirmed that pC5.22-3 directs expression of a secreted protein and established that the protein expressed by the COS-1 cells displays a broad distribution of species ranging in size between 20 and 30 Kd, a pattern typical of many of the interleukins and cytokines (Fig 2).

Sequence analysis of pC5.22-3. The nucleotide sequence of the cDNA from pC5.22-3 contains a single long open reading frame of 432 nucleotides encoding a 144-amino acid protein with a calculated molecular mass of 16 Kd (Fig 3A). The amino terminal portion of this protein includes a stretch of hydrophobic amino acids typical of secreted molecules. The sequence also contains four potential sites for asparagine-linked glycosylation. Carbohydrate modification is consistent with the extensive size heterogeneity observed with the COS cell-derived protein. In addition, the 3’ noncoding region of the cDNA contains four repeats of the ATTTA sequence observed in many cytokine and oncogene gene noncoding regions that are important in controlling the stability of the respective mRNAs.

A computer search for relatedness of pC5.22-3 to known proteins revealed that this cytokine shares extensive homology with the sequence of a newly identified murine growth factor for helper T-cell clones designated T-cell growth factor P40 (Fig. 3B). In the computer-generated alignment, all 10 cysteine residues found in the sequence of P40 matched cysteine residues of the protein encoded by pC5.22-3; the remaining cysteine in the latter sequence is found in the signal peptide. Thus, the two molecules are likely to share common tertiary structural elements. Overall, the sequences are 56% identical and 67% identical at the amino acid and nucleotide levels, respectively. These results clearly indicate that the two molecules are related and that pC5.22-3 most likely represents the human homologue of the murine T-cell growth factor P40. The difference in protein size between the murine and human homologues can be explained by the various extents of glycosylation in different cell types.

Expression of the human P40 gene. RNA blot analysis revealed that the human P40 transcript expressed by C5MJ2 cells is approximately 0.8 kilobases long (Fig 4). A transcript of similar size was also observed with mRNA preparations from PMA/PHA-stimulated human peripheral blood lymphocytes and from other human HTLV-transformed T-cell lines (CIOMJ2 and Mo), but not in mRNA preparations from the gibbon ape T-cell line UCD-144-MLA. The transcript in the HTLV-transformed T-cell lines was constitutively expressed, but the level was somewhat increased after lectin stimulation.

**DISCUSSION**

Study of the growth factor responsiveness of the human megakaryoblastic leukemic cell line, M07E, led us to the identification of a novel human cytokine that is most likely the human homologue of the murine T-cell growth factor, P40. In the mouse system, P40 was identified based on its ability to support the growth of certain helper T-cell clones in the absence of antigen or antigen-presenting cells, a property very distinct from other T-cell growth factors such as IL-2 or IL-4. In addition, murine P40 was shown to be highly specific for CD4+ T cells and was incapable of stimulating growth of cytotoxic T cells or a variety of factor-dependent
**Fig 3.** Analysis of the sequence of pC5.22-3. (A) The complete nucleotide sequence of pC5.22-3. The predicted amino acid sequence encoded by the cDNA is indicated below the nucleotide sequence in single letter code. The potential sites of asparagine-linked glycosylation are indicated by boxes around the appropriate amino acids. (B) Comparison of the predicted amino acid sequence from pC5.22-3 with that of the murine P40 is shown with identity indicated by solid lines. The conserved cysteine residues are indicated with closed triangles.
factor P40 and the human homologue we report as murine and human IL-9, respectively. Molecular cloning of the human P40/IL-9 will now facilitate analysis of the biological properties of this new cytokine.

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