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Synthesis of Membrane-Bound Colony-Stimulating Factor 1 (CSF-1) and Downmodulation of CSF-1 Receptors in NIH 3T3 Cells Transformed by Cotransfection of the Human CSF-1 and c-*fms* (CSF-1 Receptor) Genes

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NIH 3T3 cells cotransfected with the human c-*fms* proto-oncogene together with a 1.6-kilobase cDNA clone encoding a 256-amino-acid precursor of the human mononuclear phagocyte colony-stimulating factor CSF-1 (M-CSF) undergo transformation by an autocrine mechanism. The number of CSF-1 receptors on the surface of transformed cells was regulated by ligand-induced receptor degradation and was inversely proportional to the quantity of CSF-1 produced. A tyrosine-to-phenylalanine mutation at position 969 near the receptor carboxyl terminus potentiated its transforming efficiency in cells cotransfected by the CSF-1 gene but did not affect receptor downmodulation. CSF-1 was synthesized as an integral transmembrane glycoprotein that was rapidly dimerized through disulfide bonds. The homodimer was externalized at the cell surface, where it underwent proteolysis to yield the soluble growth factor. Trypsin treatment of viable cells cleaved the plasma membrane form of CSF-1 to molecules of a size indistinguishable from that of the extracellular growth factor, suggesting that trypsinlike proteases regulate the rate of CSF-1 release from transformed cells. The data raise the possibility that this form of membrane-bound CSF-1 might stimulate receptors on adjacent cells through direct cell-cell interactions.

The macrophage colony-stimulating factor CSF-1 (M-CSF) is required for the growth, differentiation, and survival of hematopoietic cells of the mononuclear phagocyte series (28). In adult animals, the growth factor is produced by mesenchymal cells and binds to a single class of high-affinity receptors expressed on mature monocytes, macrophages, and their committed progenitors (9, 10, 30). Apart from its role in hematopoiesis, CSF-1 is produced in the uterus of pregnant mice (2) and may stimulate receptors expressed on placental trophoblasts (19). The CSF-1 receptor appears to be identical to the product of the c-*fms* proto-oncogene (24) and is one of a family of proto-oncogene products and growth factor receptors that exhibit tyrosine-specific protein kinase activity.

CSF-1 is a glycosylated polypeptide homodimer assembled through interchain disulfide bonds (5, 29). A 1.6-kilobase (kb) human CSF-1 cDNA, molecularly cloned from phorbol ester-treated pancreatic carcinoma cell line, is predicted to encode a 26-kilodalton (kDa) polypeptide containing two canonical sites for addition of asparagine (N)-linked oligosaccharide chains (14). Exclusive of an amino-terminal signal peptide, the nucleotide sequence of this cDNA clone specifies a 224-amino-acid polypeptide which contains a potential hydrophobic membrane-spanning region comprising residues 166 to 188. This hydrophobic sequence is followed by a series of basic amino acids (Arg-Trp-Arg-Arg-Arg) typical of membrane "stop transfer" signals (23). These features suggest that the CSF-1 polypeptide is immobilized in the membrane of the endoplasmic reticulum (ER) during its synthesis, with its glycosylated amino-terminal domain (residues 1 to 165) in the ER cisterna and its carboxyl-terminal domain (residues 189 to 224) in the cytoplasm. The two potential sites for addition of N-linked oligosaccharides as well as seven cysteine residues are clustered within the amino-terminal domain, whereas no cysteines or predicted N-linked glycosylation sites are found in the putative cytoplasmic portion.

Using the 1.6-kb CSF-1 cDNA as a probe, longer species of CSF-1 mRNA up to ca. 4 kb in length have been detected. Since CSF-1 is encoded by a unique gene (14), these mRNAs appear to result from differential processing of primary transcripts. The 4-kb CSF-1 mRNA is the predominant species detected in a variety of CSF-1-producing cells and encodes a 554-amino-acid polypeptide. The latter includes the predicted amino-terminal domain of the 1.6-kb clone but contains an additional segment of 298 amino acids interposed upstream of the presumptive membrane-spanning sequence (16, 32). Expression of either the 1.6- or 4-kb CSF-1 cDNA in mammalian cells leads to the synthesis of a biologically active extracellular growth factor. The secreted CSF-1 polypeptide is considerably smaller than either of the predicted primary translation products and is presumed to be derived from the amino-terminal portion of its precursors by proteolysis (14, 32).

Cotransfection of NIH 3T3 cells with retroviral vectors containing the human c-*fms* and 1.6-kb CSF-1 cDNAs induces cell transformation and tumorigenicity (21). Consistent with an autocrine mechanism of transformation, we now show that these cells exhibit an increased rate of turnover of

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the c-*fms* gene product and express low numbers of cell surface CSF-1 receptors as a consequence of ligand-induced receptor downmodulation. Single-cell subclones constitutively expressing high levels of CSF-1 have allowed direct analysis of its synthesis and processing. The 224-amino-acid precursor is produced as a membrane-bound glycoprotein which is rapidly dimerized and transported to the plasma membrane. Soluble CSF-1 is released from the cell surface by proteolysis of the membrane-associated form.

MATERIALS AND METHODS

Cell lines and culture conditions. All cell lines were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, and antibiotics. NIH 3T3 cell lines transfected with retroviral DNA constructs containing the human c-*fms* or 1.6-kb CSF-1 cDNA or both have been described previously (21). Cells expressing the wild-type or mutant c-*fms* gene product (the latter containing a phenylalanine-for-tyrosine substitution at position 969, four amino acids from the carboxyl terminus) form colonies in agar only in the presence of human recombinant CSF-1 and are nontumorigenic in nude mice. By contrast, cells cotransfected with the c-*fms* and CSF-1 genes undergo morphologic transformation, form colonies in semisolid medium in the absence of exogenous CSF-1, and are tumorigenic in nude mice. When cotransfected with the CSF-1 gene, c-*fms*(Phe-969) is more efficient than c-*fms*(Tyr-969) in inducing foci of transformed cells (21). Transformed cells were seeded in agar, and colonies derived from single cells were grown and tested for CSF-1 production by either radioimmunoassay or bioassay. Individual subclones released different quantities of CSF-1, ranging from 10^3 to 10^4 U/ml of culture medium per 24 h. A cell line expressing high levels of extracellular CSF-1 was used for biochemical analyses of CSF-1 synthesis and processing.

Antisera and flow cytometric analyses. Rabbit antisera to a recombinant v-*fms*-coded polypeptide expressed in bacteria (7) were used to immunoprecipitate metabolically labeled c-*fms*-coded glycoproteins. These antisera precipitate human CSF-1 receptors expressed in peripheral blood monocytes, human HL-60 cells induced to monocyte differentiation by phorbol esters, and the human choriocarcinoma cell lines JEG and BeWo (19), but do not react with extracellular epitopes of the CSF-1 receptor expressed on the surfaces of viable cells. To raise antisera to the amino-terminal extracellular domain of the human c-*fms*-coded glycoprotein, tumorigenic NIH 3T3 cells transformed by the Harvey *ras* oncogene were cotransfected with a retroviral construct containing c-*fms* cDNA together with pSV2*neo*, a plasmid encoding resistance to neomycin (25). The transfected cells were selected in G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.), and single-cell subclones derived in semisolid medium were grown and tested for expression of the c-*fms*-coded glycoprotein by an assay for tyrosine kinase activity in immune complexes (18). Cells from a representative subclone were inoculated subcutaneously into 10-day-old syngeneic mice (5×10^6 cells per animal) and formed palpable tumors within 3 weeks of injection. Tumored animals mounted an immune response to the injected cells and produced specific antibodies to the c-*fms*-coded glycoprotein. Metabolic radiolabeling experiments and immune complex kinase assays demonstrated that the sera from two such animals reacted specifically with the c-*fms*-coded glycoprotein expressed on normal human peripheral blood monocytes or in NIH 3T3 cell lines transfected with the c-*fms* gene. The latter sera were used for flow cytometric analysis of c-*fms* expression on live cells by techniques described previously to study cell surface expression of the v-*fms* gene product (22).

Antisera to CSF-1, prepared as described previously (26), were used to quantitate both membrane-bound CSF-1 and CSF-1 binding sites by analogous procedures. Conditioned culture medium containing 1000 U of human recombinant CSF-1 per ml was used for flow cytometric analysis. The experiments were performed with CSF-1 produced in monkey COS cells, generously provided by Steven C. Clark (Genetics Institute, Cambridge, Mass.), or with CSF-1 released from NIH 3T3 cells transfected with the 1.6-kb human CSF-1 cDNA (14). To measure CSF-1 binding sites, cells grown in the presence or absence of human recombinant CSF-1 were harvested by incubation in phosphate-buffered saline containing 0.6 mM EDTA, washed, and incubated for 30 min at 4°C with an excess of human recombinant CSF-1. This procedure results in saturation of CSF-1 receptors under temperature conditions in which internalization of receptor-ligand complexes is not detectable (10). The cells were incubated with antisera to CSF-1 (30 min at 4°C), followed by incubation with a second fluoresceinated antibody that reacts with anti-CSF-1 gamma globulin prior to flow cytometric analysis.

Cell surface radioiodination. Subconfluent cultures grown in plastic tissue culture dishes (100 mm diameter) were radioiodinated with 1 mCi of ^{125}I (Amersham Corp., Arlington Heights, Ill.) and lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) as described previously (19). After removal of the labeling medium and washing in DMEM, the cells were either directly harvested by detergent lysis or incubated in fresh complete medium for various periods of time prior to analysis. The medium was recovered and clarified by centrifugation prior to analysis of released CSF-1 molecules. CSF-1 molecules in the medium or in detergent cell lysates were recovered by immunoprecipitation and analyzed by electrophoresis in denaturing polyacrylamide gels. In experiments designed to test the sensitivity of membrane-bound CSF-1 to proteolysis, iodinated cells were incubated with 100 μg of trypsin (Cooper Biomedical, Malvern, Pa.) per ml for 10 min at 4 or 22°C prior to analysis.

Other analytical procedures. Metabolic labeling with [^{35}S]methionine, cell lysis, immunoprecipitation, and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) were performed as described in detail elsewhere (1, 22). Receptor turnover studies were performed with conditioned medium from NIH 3T3 cells transfected with a retroviral construct (21) containing a 1.6-kb human CSF-1 cDNA (14) or with conditioned medium from monkey COS cells expressing a 4-kb CSF-1 cDNA clone (Genetics Institute, Cambridge, Mass.). Similar results were obtained in both cases. All lysis buffers contained 1 mM phenylmethylsulfonyl fluoride and 2% Aprotinin (Sigma Chemical Co.) as protease inhibitors. The apparent molecular weights of the immunoprecipitated products on denaturing gels were determined by comparison with the mobilities of known protein standards run in parallel.

RESULTS

Cells transformed by the c-*fms* and CSF-1 genes exhibit accelerated receptor turnover. The murine CSF-1 receptor expressed on the macrophage plasma membrane is rapidly internalized and degraded after exposure of the cells to CSF-1 (10, 31). To determine whether mouse NIH 3T3 cells

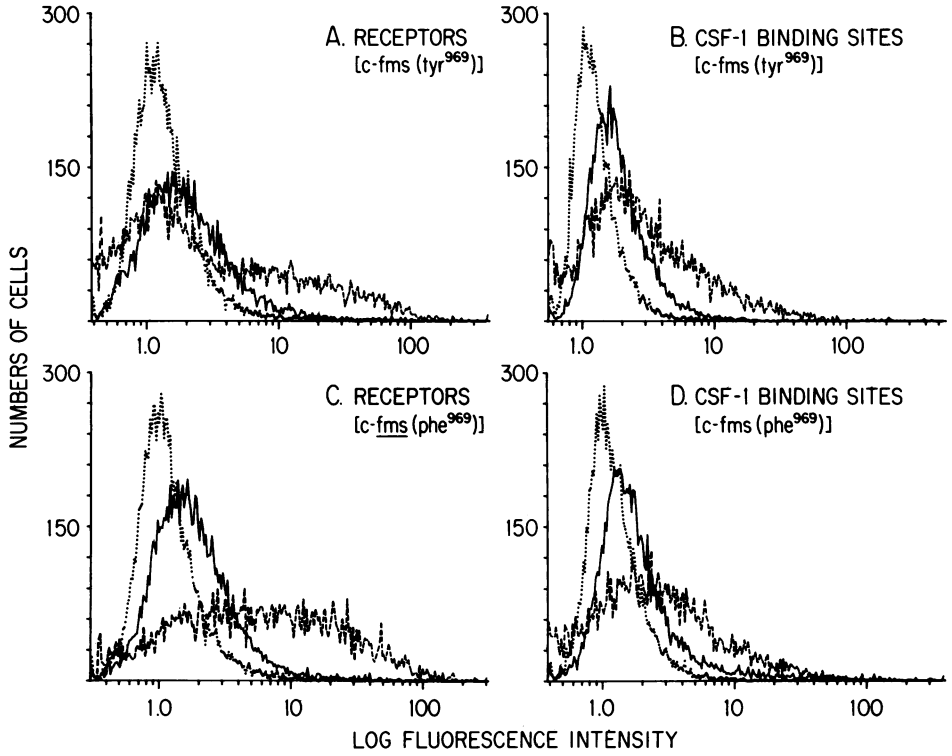

FIG. 1. CSF-1-induced downmodulation of the c-*fms* gene product (CSF-1 receptor) assayed by fluorescence-activated flow cytometry. NIH 3T3 cells were transfected with either the wild-type human c-*fms*(Tyr-969) gene (A and B) or a mutant c-*fms*(Phe-969) allele (C and D). Cell surface CSF-1 receptors (A and C) were detected by specific fluorescence with a mouse antiserum that reacts with the human c-*fms*-coded glycoprotein. The antiserum to the c-*fms* gene product detects both occupied and unoccupied receptors at the cell surface. Unoccupied CSF-1 binding sites (B and D) were determined by incubating the cells with recombinant human CSF-1 for 30 min at 4°C and then measuring specific fluorescence after addition of an antiserum to the growth factor. Without incubation at 4°C with human recombinant CSF-1, no positive fluorescence was detected by using the antiserum to the growth factor. Each panel shows the background fluorescence profile obtained with a nonspecific control antibody (dotted line) and specific fluorescence after a 2-h preincubation of the cells at 37°C in either the absence (dashed line) or presence (solid line) of recombinant human CSF-1. The difference in fluorescence profiles observed in the analyses of receptors (A and C) and CSF-1 binding sites (B and D) reflect the relative sensitivities of the two assay procedures.

expressing human CSF-1 receptors also exhibit ligand-induced receptor downmodulation, cells transfected with either a wild-type c-*fms* gene or a mutant allele encoding a phenylalanine-for-tyrosine substitution near the receptor carboxyl terminus (residue 969) were analyzed for cell surface receptors by flow cytometry. The Phe-969 mutation has been shown to potentiate the biological response to CSF-1 and augment the efficiency of transformation when c-*fms* is cotransfected with the CSF-1 gene (21). CSF-1 receptors in cell lines expressing the wild-type (Fig. 1A) or mutant (Fig. 1C) c-*fms* genes were downmodulated in response to the exogenously added growth factor. After a 2-h incubation at 37°C in medium supplemented with recombinant human CSF-1, the median fluorescence intensity with antiserum to c-*fms*-coded epitopes was less than 20% of that of cells incubated in medium lacking the growth factor. No difference in response to CSF-1 was detected between cell lines expressing c-*fms*(Tyr-969) or c-*fms*(Phe-969), demonstrating that the mutation at the receptor carboxyl terminus does not affect ligand-induced downmodulation (see below). These results were confirmed by quantitating unoccupied CSF-1 binding sites on the same cells. Cells incubated for 2 h at 37°C in the presence or absence of CSF-1 were harvested, incubated for 30 min at 4°C with human recombinant CSF-1, and then reacted with antiserum to the growth factor. Both cell lines expressed CSF-1 binding sites, which were reduced in number after preincubation at 37°C in CSF-1 conditioned medium (Fig. 1B and D). In control experiments (not shown), untransfected NIH 3T3 cells expressed neither binding sites for human CSF-1 nor epitopes reactive with the antiserum to the human c-*fms* gene product.

When assayed by metabolic radiolabeling or immune complex protein kinase reactions, cells transformed by the CSF-1 and c-*fms* genes expressed much lower levels of the mature c-*fms*-coded glycoprotein at steady state than did nontransformed cells transfected with c-*fms* genes alone. To determine whether the transformed cells constitutively downmodulated their CSF-1 receptors in response to endogenous CSF-1 production, the rates of synthesis and turnover of the c-*fms* gene product were examined in kinetic experiments. Nontransformed cell lines transfected with c-*fms* alleles alone, or transformed cells cotransfected with both the c-*fms* and CSF-1 genes, were metabolically labeled with [^{35}S]methionine for 15 min. The labeling medium was then removed, and the cells were transferred to medium containing a 100-fold excess of nonradioactive methionine in the presence or absence of human recombinant CSF-1. Detergent lysates were prepared at various time intervals, and the human c-*fms* gene products were immunoprecipitated and analyzed on denaturing polyacrylamide gels.

The human c-*fms* gene encodes an immature 130-kDa glycoprotein (gp130$^{c\text{-}fms}$) that undergoes modification of its

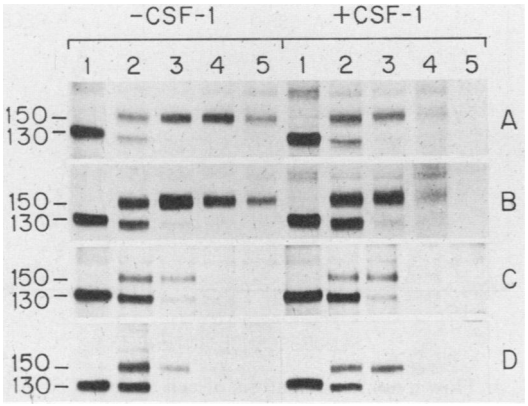

FIG. 2. Turnover of c-*fms*-coded glycoproteins in NIH 3T3 cells transfected with retroviral constructs expressing the indicated human genes: wild-type c-*fms*(Tyr-969) alone (A), mutant c-*fms*(Phe-969) alone (B), c-*fms*(Tyr-969) and CSF-1 (C), or c-*fms*(Phe-969) and CSF-1 (D). Cells were pulse labeled for 15 min with [^{35}S]methionine and then incubated in the absence (left panels) or presence (right panels) of exogenously added human recombinant CSF-1. Detergent lysates were prepared at various times after labeling and precipitated with antiserum to the c-*fms* gene product. Radiolabeled c-*fms*-coded glycoproteins were denatured in SDS, separated electrophoretically in polyacrylamide gels, and detected by fluorography. Chase intervals: time zero (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), and 4 h (lane 5). The mobilities (in kilodaltons) of the immature human c-*fms*-coded glycoprotein (gp130$^{c\text{-}fms}$) and mature cell surface form (gp150$^{c\text{-}fms}$) are noted at the left.

N-linked oligosaccharide chains during transport to the cell surface, yielding mature 150-kDa receptor molecules (gp150$^{c\text{-}fms}$) (19). In cells transfected with c-*fms* genes alone, the wild-type and mutant receptors exhibited similar rates of synthesis and turnover (Fig. 2A and B). In cells grown in the absence of CSF-1, gp130$^{c\text{-}fms}$ was converted almost completely to gp150$^{c\text{-}fms}$ within 1 h, and the mature cell surface form of the receptor turned over with a half-life of about 2 to 3 h. Under these conditions, radiolabeled gp150$^{c\text{-}fms}$ molecules were readily detected 4 h after synthesis. When the cells were grown in medium supplemented with recombinant human CSF-1, the half-life of the mature receptor was considerably diminished, with only trace amounts of gp150$^{c\text{-}fms}$ evident 2 h after synthesis. In contrast, cells transformed by c-*fms* and CSF-1 genes (Fig. 2C and D) synthesized quantities of gp130$^{c\text{-}fms}$ equivalent to those detected in cells transfected by c-*fms* alone, but exhibited greatly accelerated turnover of gp150$^{c\text{-}fms}$, even in the absence of exogenously added growth factor. Addition of CSF-1 to the medium did not further accelerate the turnover of gp150$^{c\text{-}fms}$ in the transformed cells, and no differences were detected between cells expressing either c-*fms*(Tyr-969) or c-*fms*(Phe-969). Together, the results show that (i) the steady-state levels of gp150$^{c\text{-}fms}$ are determined by receptor degradation and (ii) transformed cells coexpressing CSF-1 and c-*fms* genes synthesize receptors which undergo rapid downmodulation in the absence of exogenously added ligand.

The preceding studies were performed with selected transformed subclones secreting high levels of the human growth factor (see below). Other clones of NIH 3T3 cells cotransfected with the human c-*fms* and CSF-1 genes expressed less than 10% of the human growth factor synthesized by the high-producing lines. Metabolic radiolabeling and immunoprecipitation revealed that both classes of cells synthesized comparable amounts of gp130$^{c\text{-}fms}$. The rates of turnover of gp150$^{c\text{-}fms}$ were slower in cells producing lower levels of CSF-1, and these cells expressed proportionally higher numbers of CSF-1 receptors at their cell surface (data not shown). Nevertheless, both classes of subclones formed colonies in soft agar at equivalent efficiencies and were equally tumorigenic in nude mice. Thus, subclones producing 10-fold-lower levels of CSF-1 still synthesized sufficient quantities of the growth factor to fully transform the cells, and the rates of receptor turnover were directly proportional to the quantities of CSF-1 produced.

CSF-1 synthesized as a membrane-bound glycoprotein. Culture supernatants from single-cell clones transformed by the human c-*fms* and CSF-1 genes were screened by bioassay to identify subclones secreting high levels of human CSF-1 (ca. 10^4 U/ml of medium per day). These quantities of CSF-1 are approximately 10- to 20-fold higher than the amounts secreted by mouse L cells, a traditional source of the murine growth factor. The use of these transformed cells therefore facilitated direct biochemical analysis of the synthesis and processing of the growth factor. Cells metabolically labeled with [^{35}S]methionine for 15 min were transferred to medium containing the unlabeled precursor for various periods of time. Immunoreactive molecules were precipitated from detergent lysates of the cells and from the culture medium by using antiserum to CSF-1, and the washed immunoprecipitates were denatured and analyzed on polyacrylamide gels containing SDS in the presence or absence of disulfide-reducing agents. In parallel experiments, cells were labeled in the presence of the antibiotic tunicamycin, which prevents the addition of carbohydrate chains to canonical asparagine acceptor sites in nascent polypeptide chains (15).

The CSF-1 polypeptide labeled in the presence of tunicamycin had an apparent molecular size of 23 kDa, approximating that of the CSF-1 polypeptide predicted from its cDNA sequence (Fig. 3A, lane T). In the absence of the antibiotic, the apparent molecular size of the CSF-1 polypeptide was 31 kDa, indicating that the molecules contained N-linked oligosaccharide chains (lane 1). Since addition of a single N-linked oligosaccharide chain contributes about 3 to 4 kDa in apparent molecular mass, the difference in molecular weight between the unglycosylated and glycosylated molecules suggested that both canonical acceptor sites (Asn-X-Thr/Ser) in the polypeptide contained N-linked sugars. After 1 h of chase, the apparent molecular size of the CSF-1 glycoprotein had increased to 34 kDa (lane 2) and underwent no further detectable modifications throughout the 6-h chase period (lanes 3 to 5). The immature 31-kDa CSF-1 molecules contained N-linked sugars that were sensitive to endoglycosidase H and resistant to neuraminidase digestion, properties of mannose-rich oligosaccharides found on glycoproteins within the ER. In contrast, the 34-kDa molecules contained endoglycosidase H-resistant, neuraminidase-sensitive oligosaccharides, characteristic of glycoproteins processed within the Golgi complex (data not shown). These data are consistent with the CSF-1 cDNA sequence, which predicts the presence of an amino-terminal signal peptide that targets the CSF-1 polypeptide to the secretory compartment. When metabolically labeled cells were disrupted mechanically in the absence of detergent, all immunoprecipitable CSF-1 molecules sedimented with membranous organelles, and none were detected in the cytosol.

The newly synthesized CSF-1 polypeptides underwent rapid covalent assembly into dimers through interchain

disulfide bonds (Fig. 3B). In the absence of reducing agents, the immature form of the glycoprotein had an apparent molecular size of 62 kDa (lane 1), whereas the mature intracellular species was 68 kDa (lane 2 to 5). Assembly of CSF-1 subunits into dimers occurred rapidly since no unassembled subunits were detected in the absence of reducing agents. Even in the presence of tunicamycin (lane T), the nonglycosylated 23-kDa polypeptides formed 46-kDa homodimers. The mature 68-kDa homodimers persisted for a considerable period of time in the cells, and the majority of the pulse-labeled molecules remained cell-associated even after 6 h of chase (Fig. 3B, lanes 3 to 5). Conversely, the appearance of immunoprecipitable CSF-1 molecules in the medium occurred very slowly, with the first detectable molecules appearing 4 h after the labeling period (Fig. 3, right panels). Recovery of labeled CSF-1 in the medium was not quantitative and necessitated longer exposure of the corresponding autoradiograms (see below). The molecules released from the cells were significantly smaller than the mature intracellular glycoprotein and consisted of 44-kDa dimers composed of 22-kDa subunits. Comparison of the autoradiographic exposure times indicated that less than 5% of the cell-associated CSF-1 molecules were recovered in the medium as the processed 44-kDa growth factor.

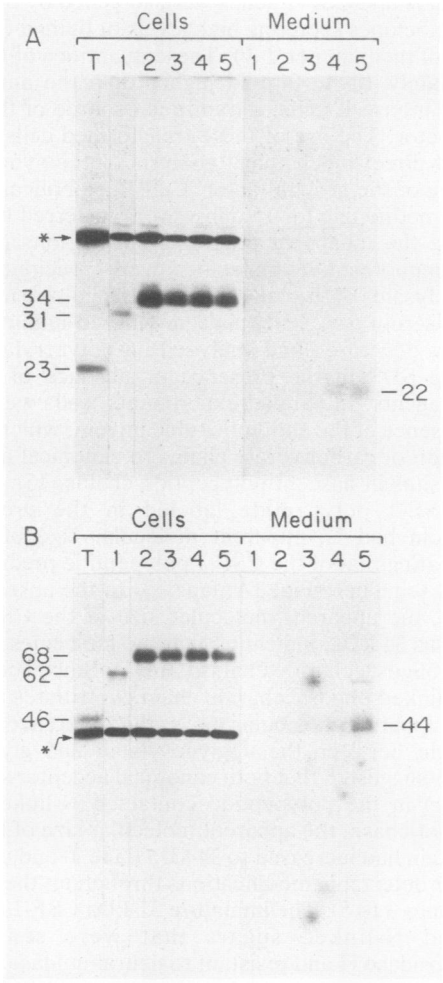

FIG. 3. Kinetics of synthesis, processing, and release of human CSF-1 in transformed NIH 3T3 cells. Cultures were pulse-labeled for 15 min with [^{35}S]methionine and then chased in medium containing excess nonradioactive methionine. Cell lysates (left panels) and culture media (right panels) were incubated with antiserum to CSF-1, and immunoprecipitates were analyzed by fluorography after SDS-polyacrylamide gel electrophoresis in the presence (A) or absence (B) of disulfide-reducing agents. Lane T, Immunoprecipitates obtained after labeling in the presence of tunicamycin, an antibiotic which blocks addition of N-linked oligosaccharide chains. Chase intervals after labeling: none (lanes 1), 1 h (lanes 2), 2 h (lanes 3), 4 h (lanes 4), and 6 h (lanes 5). The mobilities of specifically precipitated CSF-1 molecules are noted (in kilodaltons). The asterisk (*) indicates a nonspecific 43-kDa background band that was also present in precipitates prepared with nonimmune control serum. Exposure times for fluorography: cells, 4 days; medium, 20 days.

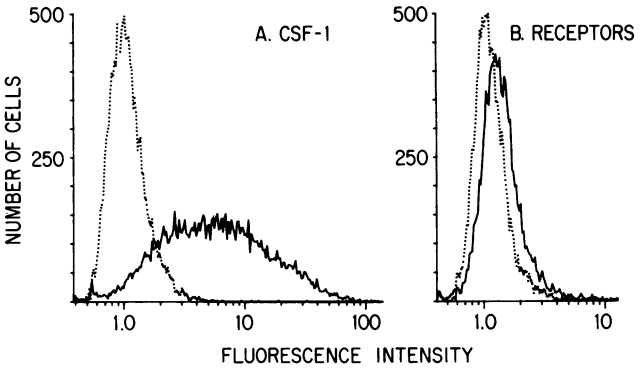

FIG. 4. Flow cytometric analysis of cell surface CSF-1 (A) and the c-*fms* gene product (CSF-1 receptor) (B) in a transformed NIH 3T3 subclone producing high levels of human CSF-1. The dotted line in each panel shows background fluorescence with a nonspecific control antibody, and the solid line indicates specific fluorescence with antiserum to CSF-1 (A) or antiserum to the human c-*fms* gene product (B). Cells transfected with the c-*fms* gene alone express about 50,000 receptors per cell, whereas cells cotransformed by c-*fms* and CSF-1 cDNA express less than 5,000 receptors per cell, at the limit of detection by flow cytometry.

The fact that CSF-1 was retarded in its release from cells and appeared as smaller molecules in the medium suggested that the mechanism of "secretion" involved proteolysis of a membrane-bound precursor. To assay for the presence of membrane-bound CSF-1, viable transformed cells were examined for the presence of CSF-1 on the plasma membrane by fluorescence-activated flow cytometry. CSF-1 epitopes were readily detected on the cell surface, whereas a control isotype-matched antibody gave a negative fluorescence profile (Fig. 4A). When the cells were incubated with recombinant human CSF-1 at 4°C and retested for CSF-1 antibody binding, no increase in fluorescence intensity was observed, suggesting that the cells lacked unoccupied CSF-1 binding sites (data not shown). Although the positive fluorescence profile obtained with the anti-CSF-1 antibody could have been due to occupied receptors, flow cytometric analyses with an antiserum to the c-*fms* gene product detected very few receptors on the transformed cells (Fig. 4B). Thus, the transformed cells expressed high levels of membrane-bound CSF-1 but only low numbers of cell surface CSF-1 receptors.

To determine the nature of the plasma membrane-bound CSF-1 molecules, viable cells were radioiodinated with lactoperoxidase, and CSF-1 molecules were immunoprecipitated from detergent lysates and analyzed on gels (Fig. 5A). In the absence of reducing agents (lane 2), the majority of membrane-bound CSF-1 molecules had an apparent molecular size of 68 kDa, identical to that of the intracellular homodimer. A minor population of 56-kDa molecules was also specifically precipitated. Electrophoresis under reducing conditions revealed primarily 34-kDa subunits as well as

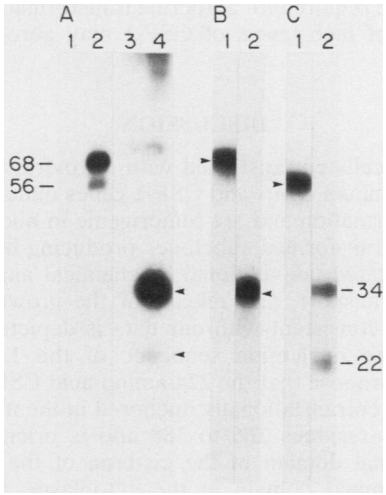

FIG. 5. Human CSF-1 molecules expressed at the surface of transfected NIH-3T3 cells. (A) Viable cells were enzymatically radioiodinated with lactoperoxidase, lysed in detergent, and incubated with a nonspecific control serum (lanes 1 and 3) or antiserum to CSF-1 (lanes 2 and 4). Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of a disulfide-reducing agent. (B and C) ^{125}I-labeled 68-kDa (B) or 56-kDa (C) CSF-1 molecules were eluted from gel slices and resubjected to SDS gel electrophoresis before (lanes 1) or after (lanes 2) disulfide reduction. The mobilities of unreduced and reduced forms of the membrane-associated CSF-1 molecules are indicated (in kilodaltons) at the left and right margins, respectively.

a minor population of 22-kDa molecules similar in size to the extracellular CSF-1 polypeptide (lane 4). When the ^{125}I-labeled 68-kDa molecules were purified by elution from gels (Fig. 5B, lane 1), reduction generated only 34-kDa subunits (lane 2), confirming that the larger form was a disulfide-linked homodimer. In contrast, the purified 56-kDa molecules (Fig. 5C, lane 1) were found to be heterodimers composed of 34-kDa and 22-kDa chains (lane 2). The 56-kDa molecule therefore contained one proteolyzed polypeptide chain assembled through disulfide bonds to an intact 34-kDa subunit. Thus, both classes of CSF-1 molecules on the plasma membrane appeared to correspond to high-molecular-weight precursors of the extracellular 44-kDa growth factor.

CSF-1 released from the plasma membrane by proteolytic cleavage. The kinetics of CSF-1 release from the plasma membrane were studied by radioiodinating live cells and then incubating them in culture medium for various periods of time. CSF-1 molecules were immunoprecipitated from detergent lysates of the cells or from the medium and subjected to electrophoretic analysis under both nonreducing and reducing conditions. The membrane-associated 68-kDa homodimer and the 56-kDa heterodimer turned over at the cell surface during the course of several hours (Fig. 6). Soluble 44-kDa homodimers of secreted CSF-1 were detected in the medium by 1 h after labeling. As in metabolic labeling experiments, quantitative conversion of the membrane-associated forms to the soluble growth factor was not observed in these cells. This could be due to several nonexclusive mechanisms. First, CSF-1 could be bound by c-*fms*-coded glycoproteins and degraded after receptor-mediated endocytosis. This possibility is consistent with the accelerated rate of turnover of the c-*fms* gene product in these cells (Fig. 2C and D) and the presence of only low numbers of cell surface CSF-1 receptors at steady state (Fig. 4B). Alternatively, unproteolyzed CSF-1 molecules at the cell surface might independently gain access to endosomes and undergo subsequent intracellular degradation. Finally, further proteolysis might degrade soluble, extracellular CSF-1 to forms no longer precipitated by the antibody. We favor the latter mechanisms, since preliminary kinetic analyses performed with NIH 3T3 cells containing amplified CSF-1 genes but lacking receptors also indicate that membrane-bound CSF-1 is not quantitatively converted to immunoprecipitable extracellular molecules.

Although the protease(s) responsible for CSF-1 release is not known, trypsin treatment of viable mouse L cells can generate biologically active CSF-1 (4). Transformed NIH 3T3 cells were therefore radioiodinated and incubated with 100 μg of trypsin per ml under various conditions. The cells were then lysed in the presence of trypsin inhibitors and immunoprecipitated with antibody to CSF-1. Incubation with trypsin for 10 min at 4°C converted a proportion of the membrane-bound molecules to 44-kDa CSF-1 homodimers (Fig. 7, lanes 2), whereas a 10-min incubation at 22°C digested all of the precursor forms (lanes 3). The subunits released from the 56- and 44-kDa molecules after trypsin digestion (Fig. 7) were similar in size to those generated in kinetic experiments (Fig. 6) when run in parallel on the same gel (data not shown). Quantitative recovery of labeled molecules after proteolysis was not achieved, suggesting that trypsin degraded the molecules to forms which were no longer immunoprecipitable. In these experiments, the ratio of 56-kDa heterodimers to 68-kDa homodimers was increased under conditions of limited tryptic digestion (Fig. 7A, lane 2) compared with cells incubated in the absence of trypsin (lane 1). This suggests that the 56-kDa form was derived from the 68-kDa species and is the direct precursor of the processed 44-kDa molecule.

Reversion of the transformed phenotype. Since CSF-1 is

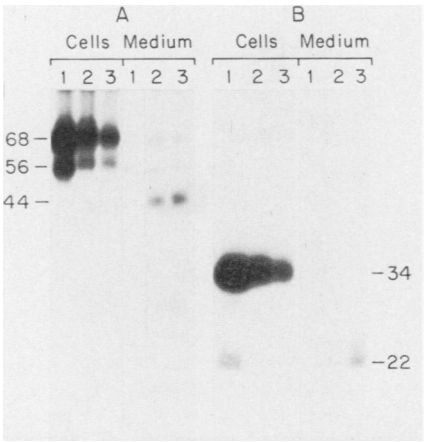

FIG. 6. Turnover of membrane-associated human CSF-1 molecules expressed at the surface of transformed NIH 3T3 cells cotransfected with the human CSF-1 and c-*fms* genes. Viable cells were radioiodinated with lactoperoxidase and then incubated in fresh medium for various periods of time. Cell lysates and harvested culture medium were immunoprecipitated with antibody to CSF-1, and ^{125}I-labeled products were detected by autoradiography after SDS gel electrophoresis under nonreducing (A) or reducing (B) conditions. Chase intervals after labeling: none, lanes 1; 1 h, lanes 2; 3 h, lanes 3. The mobilities of unreduced and reduced CSF-1 molecules are noted (in kilodaltons).

released from transformed cells by proteolysis, we tested whether trypsin inhibitors might affect the ability of cells cotransfected by the CSF-1 and c-*fms* genes to form colonies in agar. When the cells were plated in semisolid medium containing soybean trypsin inhibitor (10 mg/ml) and 2% aprotinin, no effect on the efficiency of colony formation was observed, although the average size of the colonies was somewhat reduced. In biochemical analyses, these protease inhibitors did not affect the processing of ^{125}I-labeled, membrane-bound CSF-1 to the soluble, extracellular form.

We also investigated whether neutralizing antibodies to CSF-1 might inhibit the growth of cotransfected NIH 3T3 cells in semisolid medium. Cells transfected with the c-*fms* gene alone form colonies in agar in the presence, but not the absence, of exogenously added recombinant human CSF-1 (21). In control experiments, cells transfected with c-*fms* were seeded in semisolid medium containing CSF-1 and either neutralizing antiserum to CSF-1 or control nonimmune serum. The addition of neutralizing antiserum reduced the number of agar colonies by more than 95%, and the sizes of the remaining colonies were diminished. In parallel experiments, transformed cells cotransfected by the c-*fms* and CSF-1 genes formed colonies in agar in the absence of the exogenous growth factor. Addition of high-titered neutralizing antiserum (2,000 neutralizing units/ml) to transformed subclones producing low levels of CSF-1 (ca. 10^3 U/ml per day) inhibited their colony-forming efficiency by 90%. Under the same conditions, the colony-forming efficiency of transformed subclones producing high levels of CSF-1 (ca. 10^4 U/ml per day) was reduced by only 35%. Thus, under conditions in which CSF-1 production is relatively low, an extracellular interaction between CSF-1 and its receptor appears to be required for autocrine transformation, whereas production of high levels of CSF-1 may abrogate this requirement.

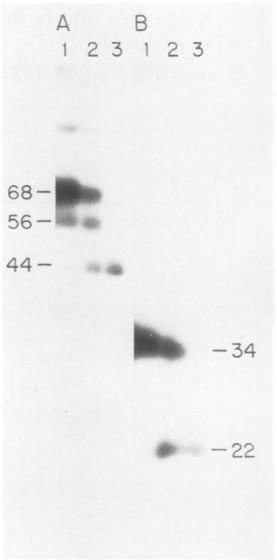

FIG. 7. Trypsin-mediated proteolysis of membrane-associated human CSF-1. Viable cells were radioiodinated with lactoperoxidase and then incubated for 10 min in phosphate-buffered saline with or without trypsin (100 μm/ml). The cells were lysed in the presence of protease inhibitors and incubated with antibody to CSF-1. Immunoprecipitated products were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing (A) or reducing (B) conditions, and ^{125}I-labeled molecules were detected by autoradiography. Incubation conditions after labeling: 22°C without protease (lanes 1), 4°C with protease (lanes 2), 22°C with protease (lanes 3). The mobilities of unreduced and reduced CSF-1 molecules are noted (in kilodaltons).

DISCUSSION

NIH 3T3 cells cotransfected with retroviral vectors containing the human c-*fms* and CSF-1 genes undergo morphologic transformation and are tumorigenic in nude mice (21). The use of transformed subclones producing high levels of CSF-1 facilitated conventional biochemical analysis of the synthesis, transport, and release of the growth factor. A model most consistent with our data is depicted in Fig. 8. Based on the nucleotide sequence of the 1.6-kb CSF-1 cDNA, we propose that the 224-amino-acid CSF-1 polypeptide chain is cotranslationally anchored in the membrane by hydrophobic residues 166 to 188 and is oriented with its amino-terminal domain in the cisterna of the ER and its carboxyl-terminal domain in the cytoplasm. Experiments with the antibiotic tunicamycin suggested that two canonical sites for N-linked oligosaccharides in the amino-terminal domain acquired carbohydrate chains during synthesis. The glycosylated subunits were rapidly dimerized to yield an immature 62-kDa form of the glycoprotein, which underwent subsequent modification of its N-linked oligosaccharide chains during transport to the plasma membrane. The mature 68-kDa glycoprotein was exteriorized on the cell surface and was sequentially processed by proteolysis to yield a 56-kDa membrane-bound heterodimeric intermediate and, ultimately, the soluble 44-kDa extracellular growth factor lacking transmembrane-spanning segments. The soluble form of human CSF-1 released from transfected NIH 3T3 cells was similar in size to a form of the protein originally purified from human urine (6).

The 68-kDa CSF-1 homodimers were expressed at the surface of transformed cells and were readily detected by fluorescence-activated flow cytometry or lactoperoxidase-catalyzed cell surface iodination. In cells producing high levels of CSF-1, few CSF-1 receptors were detected at the surface by flow cytometry, and these underwent greatly accelerated turnover compared with receptors expressed in NIH 3T3 cells transfected with the c-*fms* gene alone. No 44-kDa CSF-1 homodimers were precipitated from cell lysates, indicating that the ligand remains membrane bound until it is externalized at the cell surface. Since the steady-state level of cell surface CSF-1 molecules exceeded the number of unoccupied receptors on the plasma membrane, the local concentration of CSF-1 released by proteolysis may have been sufficiently high to allow its efficient binding to unoccupied receptors. Because both CSF-1 and its receptor are synthesized as integral transmembrane glycoproteins, receptor-ligand interactions within the secretory compartment of transformed cells are probably subject to topological restraints. However, an intracellular interaction between CSF-1 and its receptor in the secretory compartment cannot be excluded. Under conditions in which CSF-1 production was relatively high, interference of receptor-ligand interactions and reversion of the transformed phenotype with an exogenous neutralizing antiserum were not possible. Studies with other transformed subclones producing much less CSF-1 indicated that lower levels of receptor occupancy were also sufficient to render a complete biological response, leading to transformation and tumorigenicity. In contrast to transformed subclones producing high levels of CSF-1, the latter subclones were inhibited in forming colonies in agar in the presence of neutralizing antiserum to CSF-1, suggesting

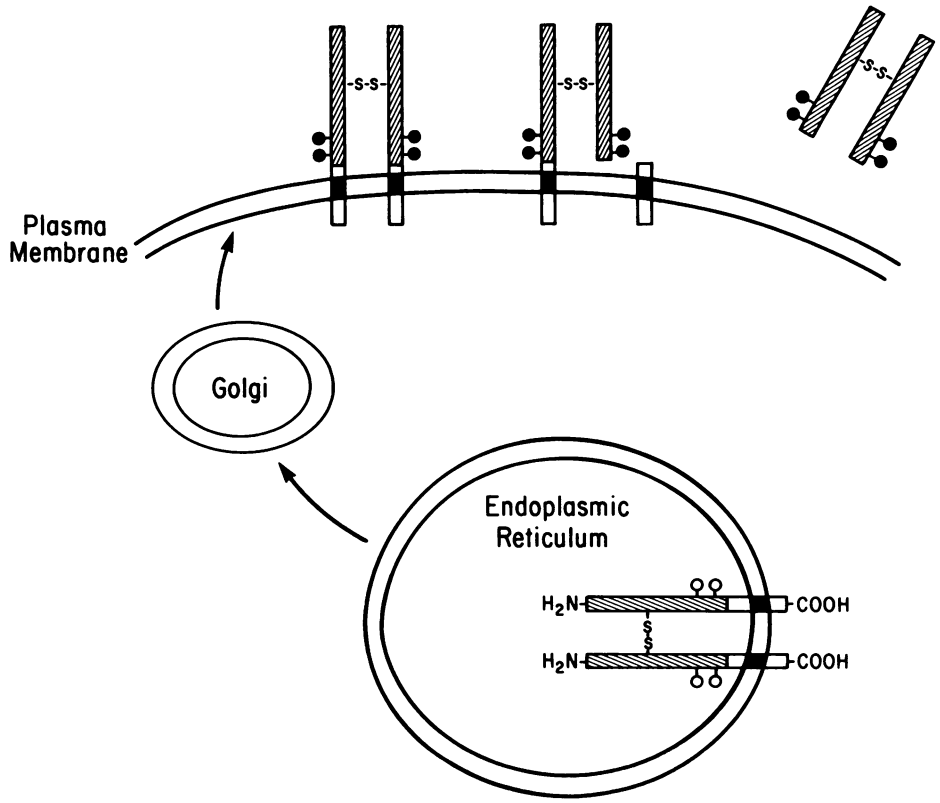

FIG. 8. Model for synthesis and processing of the product of a 1.6-kb CSF-1 cDNA. Nascent polypeptides are directed to the membrane of the ER by an amino-terminal signal sequence (not shown) and cotranslationally glycosylated with high-mannose-containing oligosaccharides (○) at two canonical sites for addition of asparagine N-linked sugars. The primary translation product is anchored in the membrane by a hydrophobic stop transfer segment near the carboxyl terminus and is rapidly assembled into disulfide-linked dimers. The molecules are transported through the Golgi complex, where N-linked oligosaccharides are processed to complex carbohydrate chains (●). Plasma membrane-associated CSF-1 precursors are cleaved by sequential proteolysis to generate the soluble extracellular growth factor (hatched areas).

that under these conditions, release of CSF-1 from the cell surface was required for autocrine transformation.

The ability of antibodies to revert the transformed phenotype has been tested in three other autocrine systems. Expression of the cloned transforming growth factor α (TGFα) gene in Rat-1 fibroblasts induces cell transformation by binding to EGF receptors expressed in the same cells (20). In this case, antibodies to the growth factor were found to reverse the transformed phenotype and inhibited the growth of these cells in semisolid medium. Transduction of the v-*sis* gene, encoding the B chain of platelet-derived growth factor (PDGF), also leads to transformation by an autocrine mechanism, and antibodies to PDGF reverted the transformed phenotype in some cases but not in others (3, 12, 13). However, an artificially anchored form of the v-*sis* protein transformed NIH 3T3 cells at an efficiency nearly as high as that observed with the wild-type v-*sis* product, suggesting that interactions between the growth factor and its receptor could occur intracellularly (11). Insertion of the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene into factor-dependent FDC-P1 myeloid cells led to factor independence and tumorigenicity, but neutralizing antiserum to GM-CSF had no effect on the growth of the factor-producing cell lines (17). The ability to antagonize the growth factor response by antibody may therefore reflect differences in neutralizing titer or affinity, the relative sensitivity of cells to different levels of receptor occupancy, or differences in the mechanism of growth factor-receptor interactions.

Release of CSF-1 into the culture medium must be regulated by proteases, but the enzymes responsible for processing the membrane-bound growth factor have not yet been identified. Cifone and Defendi (4) first showed that trypsin treatment of viable mouse L cells released a biologically active macrophage growth factor identical to CSF-1 (27). We found that trypsin would degrade the cell-associated 68-kDa CSF-1 homodimer to 44-kDa molecules that corresponded in size to those released from the cells. In this tissue culture system, either active proteases or activators of serum proenzymes must be synthesized by CSF-1-producing NIH 3T3 cells. In inflammatory reactions, proteases or activators released by participating cells might similarly be important in regulating CSF-1 release from fibroblasts, thereby enhancing the activity and survival of tissue macrophages. Particularly attractive candidates include products such as tissue plasminogen activator that are released by macrophages themselves (8) and could be involved in stimulating macrophage function by inducing local CSF-1 production. An important caveat in extrapolating our findings to physiologic systems is that many CSF-1-producing cells synthesize a

larger CSF-1 precursor (16, 32). Although nucleotide sequencing analysis predicts that this form of CSF-1 is also anchored in membranes during synthesis, the efficiency and site(s) of proteolytic processing might differ from those of the CSF-1 precursor used in our studies.

Because CSF-1 is a membrane-bound growth factor, its effects under certain circumstances might be mediated by cell-cell interactions. This may potentially be of physiologic importance in hematopoiesis, in which stromal cells in the bone marrow synthesize CSF-1 and stimulate committed receptor-bearing progenitors of mononuclear phagocytes. If the sites of proteolytic cleavage within the 224-amino-acid membrane-bound CSF-1 precursor can be identified and eliminated by appropriate mutagenesis procedures, it should be possible to determine whether presentation of cell-associated CSF-1 to receptor-bearing cells can elicit a mitogenic response.

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