MOLECULAR AND CELLULAR BIOLOGY, Nov. 1988, p. 5026–5034
0270-7306/88/115026-09$02.00/0

Vol. 8, No. 11

Differential Processing of Colony-Stimulating Factor 1 Precursors Encoded by Two Human cDNAs

CARL W. RETTENMIER* AND MARTINE F. ROUSSEL

Department of Tumor Cell Biology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, Tennessee 38105

Received 25 March 1988/Accepted 14 June 1988

The biosynthesis of macrophage colony-stimulating factor 1 (CSF-1) was examined in mouse NIH-3T3 fibroblasts transfected with a retroviral vector expressing the 554-amino-acid product of a human 4-kilobase (kb) CSF-1 cDNA. Similar to results previously obtained with a 1.6-kb human cDNA that codes for a 256-amino-acid CSF-1 precursor, the results of the present study showed that NIH-3T3 cells expressing the product of the 4-kb clone produced biologically active human CSF-1 and were transformed by an autocrine mechanism when cotransfected with a vector containing a human c-*fms* (CSF-1 receptor) cDNA. The 4-kb CSF-1 cDNA product was synthesized as an integral transmembrane glycoprotein that was assembled into disulfide-linked dimers and rapidly underwent proteolytic cleavage to generate a soluble growth factor. Although the smaller CSF-1 precursor specified by the 1.6-kb human cDNA was stably expressed as a membrane-bound glycoprotein at the cell surface and was slowly cleaved to release the extracellular growth factor, the cell-associated product of the 4-kb clone was efficiently processed to the secreted form and was not detected on the plasma membrane. Digestion with glycosidic enzymes indicated that soluble CSF-1 encoded by the 4-kb cDNA contained both asparagine(N)-linked and O-linked carbohydrate chains, whereas the product of the 1.6-kb clone had only N-linked oligosaccharides. Removal of the carbohydrate indicated that the polypeptide chain of the secreted 4-kb cDNA product was longer than that of the corresponding form encoded by the smaller clone. These differences in posttranslational processing may reflect diverse physiological roles for the products of the two CSF-1 precursors in vivo.

Colony-stimulating factors have been identified by their ability to induce the production of mature hematopoietic cells from bone marrow progenitors (2, 9). The macrophage colony-stimulating factor, CSF-1, is a glycosylated polypeptide homodimer that stimulates the proliferation, differentiation, and survival of mononuclear phagocytes (28, 29). CSF-1 also enhances the effector functions of mature monocytes and macrophages, including tumoricidal activity (17), the ability to resist viral infection (8), and the production of other cytokines (10, 32). The effects of CSF-1 are mediated by its binding to a specific cell surface receptor, now known to be the product of the c-*fms* proto-oncogene (25).

Human CSF-1 is encoded by a single gene that maps at band q33.1 on the long arm of chromosome 5 (13). Alternative splicing of the primary CSF-1 transcript generates multiple mRNAs, and human cDNAs coding for two different biologically active products have been molecularly cloned (5, 7, 33). The amino acid sequences deduced from the nucleotide sequences of the cDNA clones suggest that the primary CSF-1 translation product is an integral membrane protein. A 1.6-kilobase (kb) human cDNA encodes a 256-amino-acid product consisting of an amino-terminal signal peptide, a biologically active growth factor sequence of ca. 150 to 165 amino acids, a presumptive membrane-spanning segment, and a short carboxyl-terminal tail (5). The 554-amino-acid product of a 4-kb CSF-1 cDNA includes the entire polypeptide sequence specified by the smaller clone, but unique coding sequences derived by alternative splicing contribute a segment of 298 additional amino acid residues on the amino-terminal side of the putative transmembrane segment (7, 33).

* Corresponding author.

High-level expression of cDNAs in viral vector systems has permitted a direct analysis of CSF-1 biosynthesis. When introduced into mouse NIH-3T3 cells, the 1.6-kb human cDNA directs the synthesis of a 34-kilodalton (kDa) CSF-1 glycoprotein that is rapidly assembled into 68-kDa disulfide-linked dimers (19). The latter molecules are transported to the plasma membrane where proteolytic cleavage slowly releases a soluble 44-kDa dimeric growth factor composed of 22-kDa subunits. Insertion of a termination codon upstream of the putative transmembrane segment in the coding sequence of the 1.6-kb cDNA results in the synthesis of a soluble glycosylated product that, following removal of the amino-terminal signal peptide, assembles into biologically active dimers and is directly secreted from cells (4). The truncated molecule is similar in size to the soluble growth factor generated by proteolysis of the full-length 1.6-kb cDNA product, a fact suggesting that the site of proteolysis in the membrane-bound precursor is close to the carboxyl terminus of the truncated form. To determine whether the additional coding sequences of the 4-kb cDNA affect the processing of the CSF-1 precursor, we expressed the larger human cDNA in NIH-3T3 cells. Rapid cleavage of the 4-kb cDNA product prevented the stable expression of this molecule at the cell surface and resulted in the efficient and rapid secretion of a biologically active, soluble growth factor larger than that encoded by the 1.6-kb cDNA.

MATERIALS AND METHODS

Vector constructs. Clone pcDBCSF-4 (7), consisting of a 1.8-kb insert that included the complete coding sequences of a human 4-kb CSF-1 cDNA and was cloned into a modified Okayama Berg expression vector, was kindly provided by Martha Ladner of Cetus Corporation, Emeryville, Calif. The Susan McDonough (SM) retroviral vector derived from the

SM strain of feline sarcoma virus, from which the *gag*-v-*fms* sequences were removed, has been previously described (22). The *Bam*HI cloning site of the SM vector was converted to an *Xho*I site by digestion with *Bam*HI, filling in the single-stranded overhang with the Klenow fragment of *Escherichia coli* DNA polymerase, blunt-end ligation of *Xho*I linkers, and reclosure after *Xho*I digestion. This vector construct was prepared and kindly provided by J. M. Heard. The 4-kb CSF-1 cDNA with adjacent 5′ and 3′ pcDB vector sequences was excised from pcDBCSF-4 by digestion with *Xho*I, purified by electroelution, and ligated at the *Xho*I cloning site in the modified SM vector. Restriction enzymes, *Xho*I linkers, and the Klenow fragment of DNA polymerase were purchased from New England Biolabs, Inc., Beverly, Mass. SM vectors containing inserts of the human 1.6-kb CSF-1 cDNA (5), human c-*fms* cDNA (3), and a c-*fms* gene containing a mutation substituting phenylalanine for the normal tyrosine residue at position 969 (22) have been previously described (4, 22). Vector DNAs were transfected into NIH-3T3 cells by using the calcium phosphate precipitation technique (23). For some experiments, cells were cotransfected with pSV2*neo* (26) and selected in G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.).

Digestions with glycosidic enzymes. To determine the oligosaccharide composition of radiolabeled glycoproteins, washed immunoprecipitates were incubated for 16 h at 37°C with one or more of the following enzymes in a 20-μl volume of the indicated buffer: 0.02 IU of *Vibrio cholerae* neuraminidase (Calbiochem-Behring, La Jolla, Calif.) in 50 mM sodium acetate (pH 5.5) containing 150 mM NaCl and 4 mM $CaCl_2$; 1 mU of *Diplococcus pneumoniae* endo-α-*N*-acetylgalactosaminidase (31) (*O*-glycanase; Genzyme, Boston, Mass.) in 100 mM sodium phosphate (pH 6.0) containing 10 mM dithiothreitol, 10 mM EDTA, and 1% Nonidet-P40; or 0.5 U of *Flavobacterium meningosepticum* peptide:*N*-glycosidase F (14, 30) (*N*-glycanase; Genzyme) in 100 mM sodium phosphate (pH 8.6) containing 10 mM dithiothreitol, 10 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 1% Triton X-100. For sequential digestions with multiple enzymes, the protein A-Sepharose beads with adsorbed immune complexes were centrifuged into a pellet and the buffer of the previous glycosidase was removed prior to addition of the next enzyme; in these instances, enzymes were added in the following order: neuraminidase, *O*-glycanase, and then *N*-glycanase. For digestions with *N*-glycanase, samples were heated in buffer for 30 min at 60°C prior to the addition of the enzyme.

Other analytical methods. The procedures for metabolic radiolabeling with L-[^{35}S]methionine, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (1, 23), and cell surface iodination (21) are described in detail in the references cited. For metabolic labeling in the presence of tunicamycin (6), cells were preincubated for 4 h in complete medium containing 2 μg of tunicamycin per ml prior to labeling and chase in the continued presence of the drug.

Membrane fractions were prepared, and protease protection experiments were performed as previously described (20). NIH-3T3 cells expressing the 4-kb human CSF-1 cDNA were pulse-labeled for 15 min with [^{35}S]methionine and homogenized in hypotonic buffer (10 mM Tris hydrochloride [pH 7.4] containing 10 mM NaCl). Sucrose was added to a final concentration of 0.25 M, and the samples were centrifuged at 1,000 × *g* for 5 min at 4°C to obtain a nuclear pellet and a postnuclear supernatant. The supernatant was then centrifuged for 30 min at 100,000 × *g* to yield a crude microsomal pellet which was suspended in 50 mM Tris hydrochloride (pH 7.6) containing 50 mM KCl and 5 mM $MgCl_2$. Aliquots were incubated for 30 min at 37°C in the absence or presence of 100 μg of L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington Biochemical Corp., Freehold, N.J.) per ml with or without detergents (1% Triton X-100–0.5% sodium deoxycholate). Soybean trypsin inhibitor (100 μg/ml) was then added, and the samples were lysed in detergent, immunoprecipitated, and analyzed by SDS-PAGE.

Immune precipitation of human CSF-1 molecules was performed with the rat YYG-106 monoclonal antibody (27) which binds a polypeptide epitope shared by the murine and human growth factors. Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) precoated with a commercial rabbit anti-rat immunoglobulin G (Organon Teknika-Cappel, Malvern, Pa.) was used as immunoadsorbant. The levels of mouse CSF-1 produced by control NIH-3T3 fibroblasts were too low to be detected by immunoprecipitation with this antibody in our radiolabeling experiments. Separate incubations with an isotype-matched rat myeloma protein were performed in parallel as a control for nonspecific precipitation.

RESULTS

Expression of human 4-kb CSF-1 cDNA in NIH-3T3 cells. Coding sequences of the human 4-kb CSF-1 cDNA were cloned into a retroviral vector consisting of a provirus from an SM strain of feline sarcoma virus from which the *gag*-v-*fms* sequences had been deleted (22). Transcriptional control elements in this vector are provided by the viral long terminal repeat. Although the vector is not rescuable by retroviral superinfection because of an apparent defect in its packaging sequences, cloned inserts are expressed at high levels in mouse NIH-3T3 fibroblasts after transfection of this vector DNA by the calcium phosphate technique. In previous studies, human c-*fms* and the 1.6-kb CSF-1 cDNAs were expressed in NIH-3T3 cells with this vector (4, 22).

Coexpression of the human c-*fms* (CSF-1 receptor) and 1.6-kb CSF-1 cDNAs transforms NIH-3T3 cells by an autocrine mechanism (22). Cotransfection of the 4-kb human CSF-1 cDNA with that of c-*fms* also yielded transformed foci (Table 1). The transforming efficiency in the cotransfection assay was equivalent for the two human CSF-1 cDNAs. Transfection of the c-*fms* cDNA or CSF-1 cDNA alone did not induce focus formation, a result demonstrating that the expression of both the CSF-1 receptor and its ligand is required for transformation. Similar to results with the 1.6-kb clone, our results show that the efficiency of transformation with the 4-kb CSF-1 cDNA increased about threefold when the cotransfection assay was performed with a c-*fms* cDNA encoding a phenylalanine residue in place of the normal tyrosine at position 969, four amino acids from the receptor carboxyl terminus. This effect appears to be due to the abrogation of negative regulation of the receptor kinase activity by elimination of tyrosine 969 (22).

Expression of the 4-kb CSF-1 cDNA in transfected cells was examined by assaying the ability of conditioned medium to promote the growth of a CSF-1-dependent mouse macrophage cell line, BAC1.2F5 (11). Culture supernatants were also tested for their ability to stimulate soft-agar colony formation by NIH-3T3 cells expressing the human c-*fms* cDNA alone (22). Because murine CSF-1 does not stimulate the human receptor, the latter assay is specific for the production of the human growth factor. Transformed NIH-

TABLE 1. Efficiency of focus formation induced by retroviral constructs containing human cDNA inserts after transfection of NIH-3T3 cells[a]

Human cDNA insert	No. of focus-forming units/μg of input DNA
4-kb CSF-1	<10
1.6-kb CSF-1	<10
c-*fms*(Tyr^{969})[b]	<10
c-*fms*(Phe^{969})[b]	<10
4 kb-CSF-1 + c-*fms*(Tyr^{969})	4.3×10^3
4 kb-CSF-1 + c-*fms*(Phe^{969})	1.1×10^4
1.6-kb CSF-1 + c-*fms*(Tyr^{969})	4.1×10^3
1.6-kb CSF-1 + c-*fms*(Phe^{969})	1.1×10^4

[a] NIH-3T3 cells were transfected in 35-mm-diameter tissue culture plates with plasmid DNAs (30 ng) containing an SM retroviral vector with coding-sequence inserts of the indicated human cDNAs. Cultures were trypsinized and split into three 60-mm-diameter plates 1 day after transfection, and foci of transformed cells were enumerated 3 weeks later. The number of foci was a linear function of the concentration of input DNA. Averages from three independent experiments are given.

[b] The c-*fms*(Tyr^{969}) cDNA includes the coding sequences of the wild-type human c-*fms* gene (3). The c-*fms*(Phe^{969}) cDNA (22) contains an engineered mutation that substitutes a phenylalanine residue in place of the normal tyrosine at position 969, four amino acids from the carboxyl terminus.

3T3 fibroblasts cotransfected with c-*fms* and the 4-kb CSF-1 cDNA expressed about 5,000 to 10,000 U of human CSF-1 per ml per day. These quantities of the human growth factor were 100-fold greater than the concentration of mouse CSF-1 constitutively produced by untransfected or mock-transfected cells (24). Nontransformed NIH-3T3 cells producing similarly high levels of the human growth factor were obtained by cotransfection of the vector containing the 4-kb CSF-1 cDNA with pSV2*neo* (26) and selection of transfected cells in G418. The latter cells were used for analyses of CSF-1 biosynthesis as described below. Identical results were obtained by using the transformed cells cotransfected with the human c-*fms* and the 4-kb CSF-1 cDNA.

Synthesis of 4-kb CSF-1 cDNA product. To identify the CSF-1 products of the 4-kb human cDNA, transfected NIH-3T3 cells were metabolically radiolabeled with [^{35}S] methionine for 2 h in the presence or absence of tunicamycin, an inhibitor of asparagine (N)-linked glycosylation. The culture medium was collected, and the cell monolayers were lysed in detergent-containing buffer. The cell lysates and medium were incubated either with a monoclonal antibody to CSF-1 or with a control myeloma protein, and the precipitated products were recovered by using protein A as immunoadsorbant and analyzed by SDS-PAGE under reducing and nonreducing conditions. Cells labeled in the presence of tunicamycin expressed a 62-kDa polypeptide that was specifically precipitated with antibody to CSF-1 (Fig. 1A, lane 1) but not with the control antibody (Fig. 1A, lane 2). The apparent molecular weight of the reduced product was in good agreement with that predicted from the primary amino acid sequence encoded by the human 4-kb CSF-1 cDNA. When labeled in the absence of tunicamycin, the immunoprecipitable product had an apparent molecular mass of 70 kDa (Fig. 1A, lane 3), a result indicating that the product underwent an addition of N-linked oligosaccharides. SDS-PAGE analysis of immune complexes from the radiolabeled cell lysates revealed several conspicuous background bands (e.g., at 200 and 43 kDa) that were also nonspecifically precipitated by the control myeloma protein (Fig. 1A, lanes 2 and 4). Immunoprecipitation of the culture medium and disulfide reduction demonstrated soluble 36- or 43-kDa (Fig. 1B, lanes 1 and 3) CSF-1 subunits in the

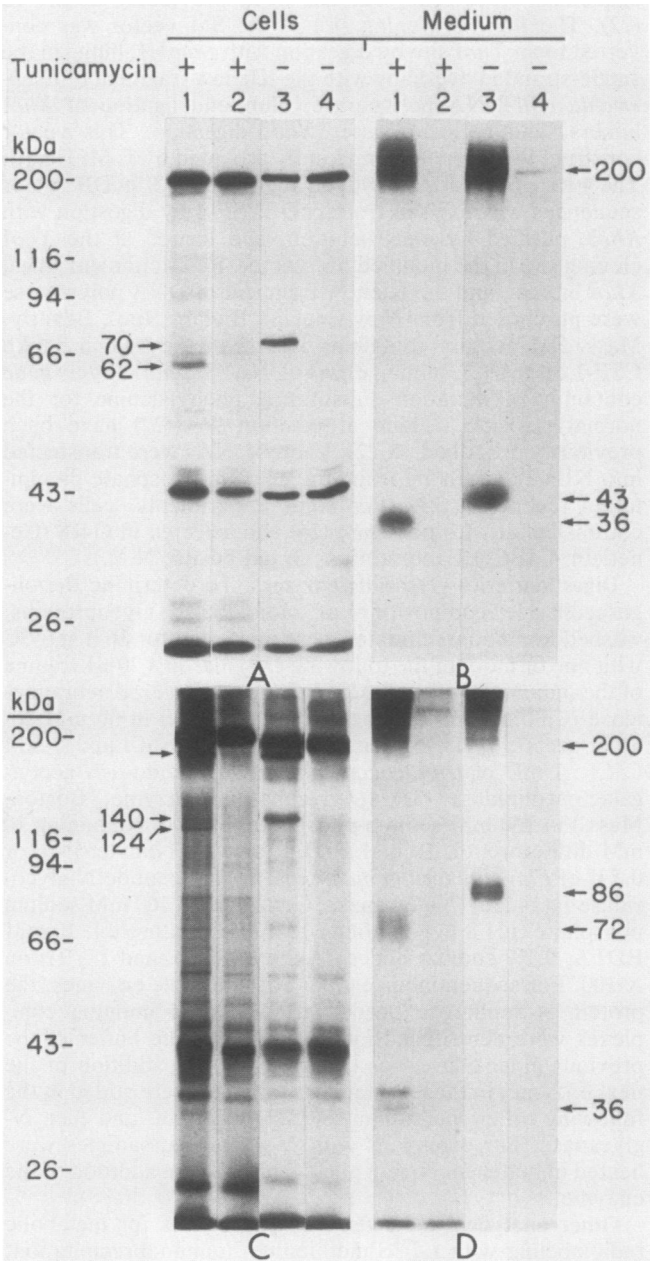

FIG. 1. Cell-associated and secreted forms of human 4-kb CSF-1 cDNA product expressed in NIH-3T3 cells. Cells were metabolically radiolabeled with 250 μCi of [^{35}S]methionine per ml for 2 h in the presence (+) or absence (−) of tunicamycin. The culture medium was collected, and cells were lysed in detergent-containing buffer. Cell lysates (A and C) and harvested culture media (B and D) were incubated with either a rat monoclonal antibody to CSF-1 (lanes 1 and 3) or control myeloma protein (lanes 2 and 4), and the precipitated products were separated by SDS-PAGE either with (A and B) or without (C and D) disulfide reduction. Radiolabeled products were detected by fluorography of the dried gels. The mobilities of specifically precipitated products (arrows) are noted at the left and right margins, and the migration of protein standards of known molecular size is indicated at the left. Exposure times for fluorography are as follows: panels A and B, 7 days for lanes 1 and 2 and 5 days for lanes 3 and 4; panels C and D, 10 days for lanes 1 and 2 and 8 days for lanes 3 and 4.

presence or absence of tunicamycin, respectively. In addition, a diffuse [^{35}S]methionine-labeled band having an apparent molecular mass of about 200 kDa was also specifically precipitated from both samples. The partial characterization of this high-molecular-weight material detected in the medium is discussed below.

When analyzed under nonreducing conditions, cell-associated CSF-1 products labeled in the presence or absence of tunicamycin had apparent molecular masses of 124 or 140 kDa (Fig. 1C, lanes 1 and 3), respectively. Only trace amounts of the monomeric subunits were detected under these conditions, a fact suggesting that the cell-associated 4-kb cDNA product was readily assembled into disulfide-linked dimers. In addition, variable amounts of material migrating at about 190 kDa were detected in the gels (Fig. 1C, arrow); this observation suggests that the 4-kb cDNA product could have been linked by disulfide bonds to other cellular proteins or might have assembled into larger oligomeric forms of CSF-1. Soluble CSF-1 dimers of 72 or 86 kDa (Fig. 1D, lanes 1 and 3) were recovered from the medium in the presence or absence of tunicamycin, respectively. The secretion of some 36-kDa monomers was also detected from cells labeled in the presence of tunicamycin (Fig. 1D, lane 1). Taken together, these results indicate that the primary translation product of the 4-kb CSF-1 cDNA (i) undergoes addition of N-linked oligosaccharides and (ii) is assembled intracellularly into disulfide-linked dimers. Moreover, because secreted CSF-1 recovered from the medium was smaller than the cell-associated form, the results further indicate that the primary translation product (iii) undergoes proteolysis to generate the soluble extracellular growth factor. These three features of the biosynthesis of the 4-kb CSF-1 cDNA product are similar to those previously observed with the product of the 1.6-kb human clone (19).

NIH-3T3 fibroblasts constitutively synthesize low levels of mouse CSF-1 (24), but the concentration of murine growth factor produced by mock-transfected cells was more than 100-fold less than that of human CSF-1 obtained from cells expressing the human 4-kb cDNA. Although the anti-CSF-1 antibody used in our experiments reacted with both the mouse and human growth factors, the amounts of murine CSF-1 synthesized in untransfected NIH-3T3 cells were too low to be detected by immunoprecipitation with this antibody in radiolabeling studies. None of the cell-associated or soluble molecules (Fig. 1, arrows), including the 200-kDa material, precipitated with the anti-CSF-1 antibody from untransfected or mock-transfected NIH-3T3 cells in control experiments. Thus, these bands appear to represent the authentic products of the 4-kb human CSF-1 cDNA or specifically coprecipitating molecules.

Evidence that the 70-kDa CSF-1 subunit is an integral transmembrane glycoprotein. An internal sequence of hydrophobic amino acids anchors the 34-kDa glycoprotein product of the 1.6-kb cDNA in the plasma membrane (19). Insertion of a termination codon upstream of this hydrophobic segment results in the synthesis of a biologically active soluble growth factor that is directly secreted from the cell (4). To determine whether this hydrophobic segment similarly serves as a transmembrane sequence for the 70-kDa glycoprotein precursor, protease protection experiments were carried out with microsomes from cells expressing the 4-kb CSF-1 cDNA. Cells pulse-labeled with [^{35}S]methionine for 15 min were mechanically disrupted in hypotonic buffer in the absence of detergent, and cytosol and membrane fractions were prepared by differential centrifugation. In preliminary experiments, all of the immunoprecipitable 70-kDa

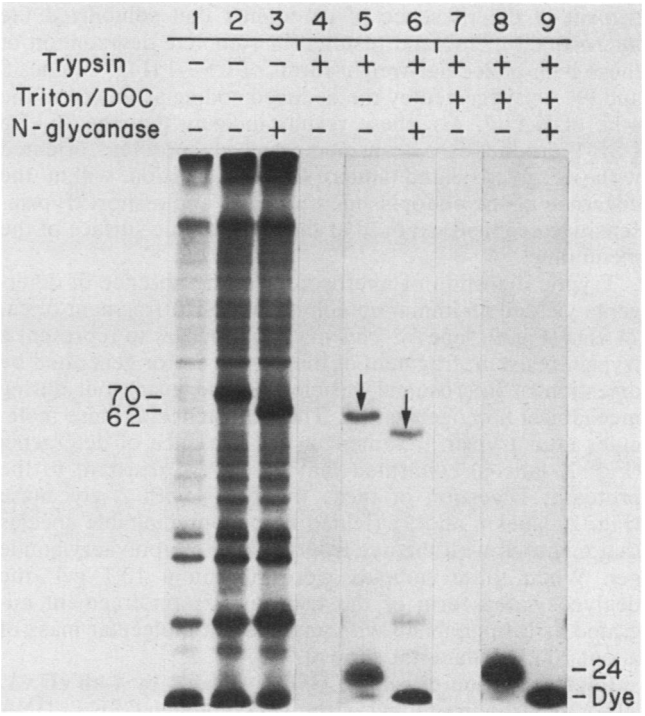

FIG. 2. Evidence that the cell-associated 70-kDa CSF-1 product of 4-kb human cDNA is an integral transmembrane glycoprotein. NIH-3T3 cells expressing the 4-kb human CSF-1 cDNA were metabolically labeled with [^{35}S]methionine for 15 min and then mechanically homogenized in hypotonic buffer in the absence of detergent. A membrane fraction was prepared by differential centrifugation, and portions were incubated for 30 min at 37°C in the absence (−) or presence (+) of trypsin (100 μg/ml) and detergents (1% Triton X-100–0.5% sodium deoxycholate [DOC]). Soybean trypsin inhibitor (100 μg/ml) was then added, and all samples were lysed in detergent and precipitated with either control myeloma protein (lanes 1, 4, and 7) or monoclonal antibody to CSF-1 (all other lanes). Immunoprecipitated products were incubated in the absence (−) or presence (+) of *N*-glycanase prior to electrophoresis under reducing conditions in a 7.5% polyacrylamide-SDS gel. The mobilities of specifically precipitated CSF-1 bands are indicated in kilodaltons at the margins. Membrane-protected proteolytic fragments of the CSF-1 precursor are indicated by arrows in lanes 5 and 6.

CSF-1 molecules were detected in the membrane fraction (data not shown). Portions of the membrane fraction were incubated at 37°C for 30 min with trypsin in the presence or absence of detergents, and after the addition of protease inhibitors, the samples were lysed and incubated with antibody to CSF-1. The immunoprecipitated products were analyzed by SDS-PAGE either before or after digestion with *N*-glycanase to remove N-linked oligosaccharides.

Digestion of the membrane-bound 70-kDa CSF-1 product (Fig. 2, lane 2) with *N*-glycanase yielded a 62-kDa species lacking N-linked oligosaccharides (Fig. 2, lane 3); the latter form comigrated with the polypeptide detected after metabolic labeling in the presence of tunicamycin (Fig. 1A). The presence only of glycosylated 70-kDa subunits and the absence of unglycosylated CSF-1 molecules after a 15-min pulse-labeling interval suggested that the rapid addition of N-linked oligosaccharides to the CSF-1 polypeptide was a cotranslational event. Digestion of microsomes with trypsin converted the 70-kDa glycosylated form to a 60- to 62-kDa molecule (Fig. 2, arrow in lane 5) that was also sensitive to *N*-glycanase (Fig. 2, arrow in lane 6). Incubation with

trypsin in the presence of detergents that solubilized the microsomal membrane resulted in complete degradation of these high-molecular-weight forms of CSF-1 (Fig. 2, lanes 8 and 9). As suggested by the deduced coding sequence of the 4-kb cDNA (7, 33), these results indicate that the 70-kDa CSF-1 product is a transmembrane glycoprotein oriented with its glycosylated amino terminal portion within the cisternae of the endoplasmic reticulum and a short trypsin-sensitive segment exposed at the cytoplasmic surface of the membrane.

Tryptic digestion of microsomes in the absence of detergents yielded an immunoprecipitable CSF-1 fragment of ca. 24 kDa (Fig. 2, lane 5). This material appears to represent a trypsin-resistant fragment of the growth factor generated by digestion of microsomal vesicles formed inside out during mechanical homogenization. The persistence of these molecules after trypsin treatment in the presence of detergents (Fig. 2, lane 8) confirmed that they were resistant to the protease. Digestion of these fragments with *N*-glycanase (Fig. 2, lanes 6 and 9) yielded immunoprecipitable species that migrated with the dye front in the 7.5% polyacrylamide gel. When these samples were run on a 10% gel, the deglycosylated form of the trypsin-resistant fragment migrated as a single band with an apparent molecular mass of about 20 kDa (data not shown).

Rapid secretion of soluble CSF-1 encoded by 4-kb cDNA. The glycoprotein product of the 1.6 kb-human CSF-1 cDNA is stably expressed on the plasma membrane of transfected NIH-3T3 cells as a disulfide-linked 68-kDa dimer composed of 34-kDa subunits (19). This molecule has a slow turnover and is inefficiently cleaved at the surface of cultured NIH-3T3 cells to release the growth factor into the medium. To determine the kinetics of the secretion of the soluble 4-kb cDNA product, transfected cells were pulse-labeled for 15 min with [^{35}S]methionine and chased for various intervals in medium containing a 100-fold excess of the nonradioactive precursor. Cell lysates and culture media were incubated with antibody to CSF-1, and the precipitated products were analyzed by SDS-PAGE under reducing conditions. The cell-associated 70-kDa CSF-1 product had a rapid turnover, with a marked reduction in the intensity of the radiolabeled band within 30 min and its virtually complete disappearance by 60 min after labeling (Fig. 3). Concurrently, 43-kDa CSF-1 subunits were detected in the medium within 30 min after labeling, and the secretion of these molecules was complete by 1 h. These results demonstrate that, in contrast to the product of the human 1.6-kb cDNA, the glycoprotein encoded by the 4-kb clone is rapidly processed and secreted. Analysis of these samples by SDS-PAGE under nonreducing conditions revealed primarily the larger dimeric forms of both the cell-associated and secreted growth factor shown in Fig. 1C and D, a result indicating that the intracellular assembly of CSF-1 subunits encoded by the 4-kb cDNA occurs rapidly after synthesis. [^{35}S]methionine-labeled 200-kDa molecules were recovered in immunoprecipitates of the chase medium (cf. Fig. 1 and 3) with the same kinetics as the recovery of the soluble 43-kDa form of the growth factor.

To determine whether the 4-kb CSF-1 cDNA product was present on the plasma membrane, viable cells were incubated with the monoclonal antibody to CSF-1 and assayed for surface expression of the epitope by fluorescence-activated flow cytometry. The CSF-1 epitope recognized by this antibody was not detected on NIH-3T3 cells expressing the 4-kb clone under conditions in which it was readily demonstrated on the plasma membrane of cells expressing the product of the 1.6-kb human cDNA. To confirm this result,

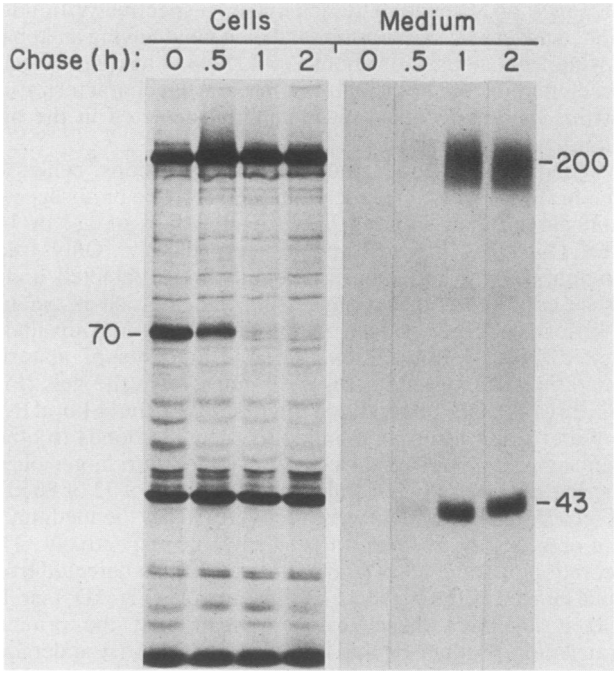

FIG. 3. Secretion kinetics of soluble CSF-1 product encoded by 4-kb human cDNA. Transfected NIH-3T3 cells were pulse-labeled for 15 min with [^{35}S]methionine. The labeling medium was then removed, and parallel cultures were incubated in medium containing a 100-fold excess of nonradioactive methionine. At the indicated times, the culture medium was collected, and the cells were lysed in detergent-containing buffer. Cell lysates and the corresponding chase medium were immunoprecipitated with antibody to CSF-1, and the radiolabeled products were separated by SDS-PAGE and detected by fluorography of the dried gel. The molecular masses of specifically precipitated bands are indicated in kilodaltons at the margins.

viable cells expressing equivalent amounts of either the human 1.6- or 4-kb CSF-1 cDNA products were enzymatically radioiodinated with lactoperoxidase and either lysed immediately or incubated in medium for an additional 3 h. Cell lysates and harvested culture medium were precipitated with either the monoclonal antibody to CSF-1 or control myeloma protein, and ^{125}I-labeled products were separated by SDS-PAGE under reducing conditions. Figure 4A demonstrates the presence of ^{125}I-labeled 34- and 22-kDa forms of CSF-1 on the surface of cells expressing the 1.6-kb human cDNA (lane 1); the latter molecule is derived from a minor population of partially cleaved, membrane-bound heterodimers consisting of one 34- and one 22-kDa subunit (19). Soluble 22-kDa subunits derived from the secreted 44-kDa homodimer were recovered from the medium of cells expressing the 1.6-kb cDNA clone (Fig. 4B, lane 1). By contrast, no ^{125}I-labeled CSF-1 products were detected on the plasma membrane or in the medium from cells expressing the 4-kb CSF-1 cDNA (Fig. 4, lanes 3). Thus, the soluble product of the 4-kb CSF-1 cDNA is rapidly secreted, and its precursor is not stably expressed on the surface of transfected NIH-3T3 cells.

Glycosylation of human CSF-1. The carbohydrate composition of the secreted forms of CSF-1 produced by the two human cDNAs was examined by digestion with enzymes that remove oligosaccharide chains. Medium conditioned by NIH-3T3 cells producing radiolabeled CSF-1 was precipitated with anti-CSF-1 antibody, and the products were

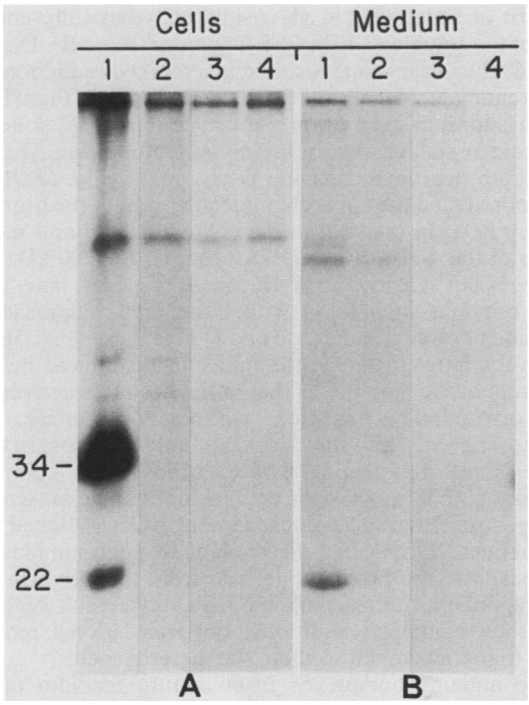

FIG. 4. Lactoperoxidase-catalyzed surface iodination of NIH-3T3 cells expressing CSF-1 cDNAs. Viable cells were enzymatically radioiodinated and then either lysed immediately (A) or incubated in medium that was collected 3 h later (B). Lanes: 1 and 2, human 1.6-kb CSF-1 cDNA; 3 and 4, human 4-kb CSF-1 cDNA. Samples were incubated with either antibody to CSF-1 (lanes 1 and 3) or control myeloma protein (lanes 2 and 4), and the precipitated ^{125}I-labeled products were separated by SDS-PAGE under reducing conditions and detected by autoradiography. The mobilities of the membrane-bound 34-kDa and soluble 22-kDa CSF-1 subunits encoded by the 1.6-kb cDNA are indicated at the left margin. No ^{125}I-labeled forms of CSF-1 were detected on the surface or from the medium of cells expressing the 4-kb human clone.

incubated with glycosidic enzymes prior to analysis on gels under reducing conditions. The soluble 22-kDa subunit encoded by the 1.6-kb cDNA contained N-linked oligosaccharides with terminal sialic acids (Fig. 5A). Treatment with neuraminidase, which removes terminal sialic acid residues, resulted in a slight decrease in the apparent molecular weight of the subunit (Fig. 5A, lane 3). Digestion with *N*-glycanase, which cleaves the complete N-linked carbohydrate moiety, yielded molecules with an apparent molecular mass of 19 kDa (Fig. 5A, lane 5). Sequential digestion with neuraminidase and *N*-glycanase similarly gave the 19-kDa product (Fig. 5A, lane 6), a result indicating that all of the sialic acid residues were present on N-linked oligosaccharides. Consistent with these results, the 22-kDa subunits were resistant to endoglycosidase H, which cleaves immature N-linked chains of the high-mannose type, but not to their processed derivatives (data not shown). The coding sequence of the 1.6-kb cDNA contains two canonical sites for N-linked glycosylation, and digestion with limiting amounts of *N*-glycanase revealed the presence of two N-linked oligosaccharides. There was no evidence for O-linked glycosylation of the 22-kDa CSF-1 subunit; the molecule was completely resistant to digestion by *O*-glycanase before (Fig. 5A, lane 2) or after incubation with either neuraminidase alone (Fig 5A, lane 4) or neuraminidase and *N*-glycanase (Fig. 5A, lane 7). Similar digestions of the 34-kDa plasma membrane-bound

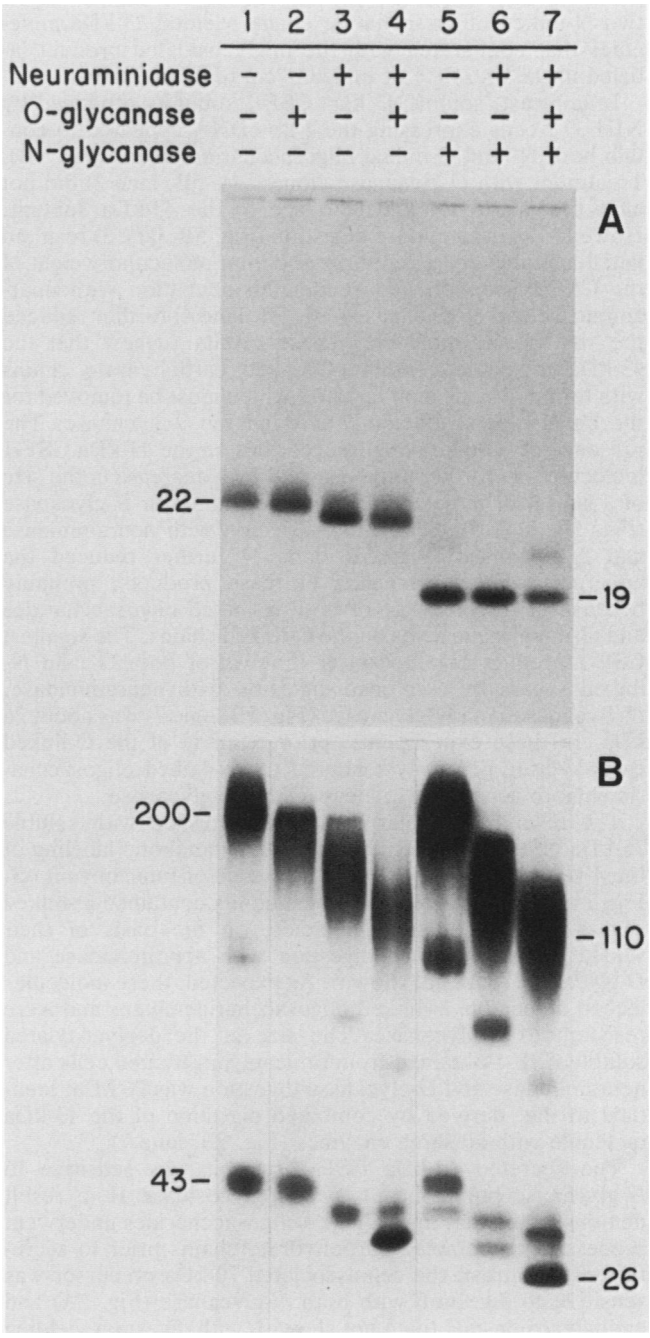

FIG. 5. Carbohydrate composition of secreted CSF-1 products encoded by the human cDNAs. (A) 1.6-kb human cDNA; (B) 4-kb human cDNA. Radiolabeled CSF-1 was obtained from the culture medium of NIH-3T3 cells expressing the corresponding human cDNA. Medium was collected 3 h after lactoperoxidase-catalyzed cell surface iodination (A) or metabolic labeling with [^{35}S]methionine (B). Samples were immunoprecipitated with antibody to CSF-1, and the immune complexes were incubated in the absence (−) or presence (+) of the indicated glycosidic enzymes, as described in Materials and Methods. Products were then separated by SDS-PAGE and detected by autoradiography of the dried gel. The mobilities of the major glycosylated and deglycosylated products are indicated at the left and right margins, respectively.

form of the 1.6-kb cDNA product revealed an identical pattern of sensitivity to glycosidic digestion; removal of the two N-linked oligosaccharide chains yielded 23-kDa molecules that comigrated with the unglycosylated product labeled in the presence of tunicamycin (data not shown).

In contrast, soluble 43-kDa CSF-1 subunits produced by NIH-3T3 cells expressing the 4-kb cDNA appeared to contain both N- and O-linked oligosaccharide chains (Fig. 5B). Treatment with *O*-glycanase alone (Fig. 5B, lane 2) did not alter the apparent molecular size of the 43-kDa subunit. However, neuraminidase digestion (Fig. 5B, lane 3) resulted in a detectable decrease in the apparent molecular weight of the CSF-1 product, and sequential incubation with neuraminidase and *O*-glycanase (Fig. 5B, lane 4) further reduced the size of the molecule. These results suggest that the 43-kDa molecule contains O-linked carbohydrate chains with terminal sialic acid residues which must be removed for the chains to be efficiently cleaved by *O*-glycanase. The presence of N-linked oligosaccharides on the 43-kDa CSF-1 molecule was further demonstrated by a decrease in the size of a subset of molecules after incubation with *N*-glycanase (Fig. 5B, lane 5). Sequential digestion with neuraminidase and *N*-glycanase (Fig. 5B, lane 6) further reduced the apparent molecular weights of these products, probably because of the cleavage of both N-linked oligosaccharides and terminal sialic acids on the O-linked chains. The smallest CSF-1 product obtained after removal of both O- and N-linked sugars by combined digestion with neuraminidase, *O*-glycanase, and *N*-glycanase (Fig. 5B, lane 7) was about 26 kDa. In these experiments, prior removal of the O-linked carbohydrate generally rendered the N-linked oligosaccharides more accessible to cleavage by *N*-glycanase.

The results of similar analyses performed with soluble 36-kDa CSF-1 subunits obtained after metabolic labeling of the 4-kb cDNA product in the presence of tunicamycin (cf. Fig. 1 and 5) suggested that the subunits contained O-linked sugars with terminal sialic acids, on the basis of their sensitivity to combined digestion with neuraminidase and *O*-glycanase (data not shown). As expected, these molecules lacked detectable N-linked oligosaccharide chains and were resistant to *N*-glycanase. The size of the deglycosylated soluble CSF-1 obtained from tunicamycin-treated cells after neuraminidase and *O*-glycanase digestion was 26 kDa, identical to that derived by combined digestion of the 43-kDa molecule with all three enzymes (Fig. 5B, lane 7).

The secreted 43-kDa CSF-1 subunit was sensitive to *N*-glycanase but resistant to endoglycosidase H, a result demonstrating that the N-linked oligosaccharides underwent processing to complex carbohydrate chains prior to secretion. By contrast, the cell-associated 70-kDa precursor was sensitive to digestion with both *N*-glycanase (Fig. 2A) and endoglycosidase H (data not shown), with digestion yielding molecules that comigrated with the 62-kDa intracellular form labeled in the presence of tunicamycin in each case. These results demonstrated that the intracellular 70-kDa precursor contained N-linked oligosaccharides of the high-mannose type, a characteristic typical of immature glycoproteins in the endoplasmic reticulum. The 70-kDa form was also completely resistant to neuraminidase and *O*-glycanase, a fact indicating that the uncleaved precursor does not undergo addition of O-linked sugars before its N-linked chains are processed to complex oligosaccharides. The results suggest that the latter carbohydrate modifications take place near the time of proteolytic cleavage and just prior to the secretion of the soluble product.

The immunoprecipitable 200-kDa molecule exhibited a pattern of sensitivity to glycosidic enzymes different from that of the soluble 43-kDa CSF-1 subunit (Fig. 5B). Digestion with *O*-glycanase alone resulted in a marked reduction in the apparent molecular size of the 200-kDa species (Fig. 5B, lane 2). In addition, incubation with neuraminidase alone (Fig. 5B, lane 3) yielded a more rapidly migrating form. Digestion with both neuraminidase and *O*-glycanase (Fig. 5B, lane 4) further reduced the apparent molecular size of the product to ca. 110 kDa. In contrast to the cell-associated and secreted forms of the 4-kb CSF-1 cDNA product, the 200-kDa molecule was not sensitive to *N*-glycanase (Fig. 5B, lane 5) even after previous incubation with either neuraminidase (Fig. 5B, lane 6) or neuraminidase and *O*-glycanase (Fig. 5B, lane 7). In the latter instance, the molecules exhibited the same electrophoretic mobility as that of the products derived from the corresponding digestions without *N*-glycanase. These results suggest that the 200-kDa molecule consists of a glycosylated polypeptide whose carbohydrate component includes both terminal sialic acid residues and exposed sugar linkages sensitive to *O*-glycanase but lacks N-linked oligosaccharides. Even after digestion with neuraminidase and *O*-glycanase, the products derived from the 200-kDa material migrated as diffuse bands in the gels, a result suggesting that they contained additional posttranslational modifications that contributed to their size heterogeneity.

The immune complexes from culture medium of [^{35}S] methionine-labeled cells (Fig. 5) contained variable amounts of a ca. 90-kDa molecule that was also nonspecifically precipitated by a control myeloma protein. Like the 200-kDa material, this background band was resistant to *N*-glycanase but sensitive to neuraminidase and *O*-glycanase, yielding a ca. 60-kDa product after combined digestion with the latter enzymes.

DISCUSSION

The 4-kb human CSF-1 cDNA encodes a polypeptide that includes all 256 amino acids specified by the 1.6-kb clone with an additional segment of 298 residues inserted on the amino-terminal side of the transmembrane segment (7, 33). The presence of these additional amino acids alters the mechanism by which the soluble CSF-1 growth factor is generated from the corresponding membrane-bound precursor. In the present study, the human CSF-1 cDNAs were cloned into a retroviral vector and expressed in NIH-3T3 cells. Very similar results are reported in the accompanying article by Manos (8a), who expressed the human 4-kb cDNA coding sequences in mouse C127 cells via a bovine papilloma virus vector. The primary translation products of both the 1.6- and 4-kb cDNAs undergo addition of N-linked oligosaccharides and rapid assembly into dimers maintained by disulfide bonds. The membrane-associated precursor encoded by the 1.6-kb clone is stably expressed at the cell surface where low levels of the soluble growth factor are slowly released by proteolytic cleavage (19). Proteolysis of the cell-associated product of the 4-kb clone takes place rapidly and results in efficient secretion of an extracellular growth factor. In contrast to the product of the 1.6-kb cDNA, membrane-bound forms encoded by the 4-kb clone were not detected at the cell surface. Moreover, the intracellular 70-kDa subunit encoded by the 4-kb cDNA contained only immature N-linked carbohydrate chains and lacked complex N- and O-linked sugars, results suggesting that oligosaccharide processing and proteolytic cleavage take place intracellularly immediately prior to the secretion of the soluble 86-kDa homodimer. Evidence in support of

this hypothesis is the previous finding that cleavage of proinsulin occurs within secretory vesicles after the molecule exits the Golgi complex (12).

The secreted CSF-1 molecules produced from the 1.6- and 4-kb cDNAs differed in both the lengths of their polypeptide chains and their carbohydrate moieties. Both processed forms contained N-linked sugars consisting of complex endoglycosidase H-resistant oligosaccharides. The N-linked carbohydrate does not appear to be required for the transport and secretion of the 4-kb cDNA product because soluble molecules lacking N-linked chains were recovered from the medium in the presence of tunicamycin. However, the soluble 43-kDa molecule encoded by the 4-kb cDNA also apparently contains O-linked carbohydrate, which was not detected in the secreted 22-kDa subunit derived from the smaller clone. Cleavage of N-linked oligosaccharides from the soluble 22-kDa molecule yielded a 19-kDa polypeptide that was very similar in size to the deglycosylated product obtained from a truncated form of CSF-1 predicted to contain 158 amino acids (4). Removal of N- and O-linked sugars from the 43-kDa subunit yielded a 26-kDa product. This result indicates that the secreted polypeptide encoded by the 4-kb cDNA is longer than that of the 1.6-kb clone and is in agreement with the finding of Wong et al. (33) that the carboxyl-terminal amino acids are derived from the inserted coding sequences in the 4-kb clone. This unique 298-amino-acid segment encoded by the 4-kb cDNA contains several serine and threonine residues that might serve as attachment sites for O-linked carbohydrate chains. Differences in the cleavage sites within the two CSF-1 precursors also appear to be responsible for the more rapid rate of secretion of the larger soluble product.

The immunoprecipitation of culture medium from NIH-3T3 cells expressing the human 4-kb cDNA with antibody to CSF-1 revealed 43-kDa subunits and a diffuse band of ca. 200 kDa. The latter molecule was apparently a heavily O-glycosylated polypeptide that lacked detectable N-linked carbohydrate. We think that the 200-kDa material is specifically associated with the 43-kDa CSF-1 molecule because it was not observed in immunoprecipitates of culture medium from untransfected cells. The 200-kDa material was also immunoprecipitated with the 4-kb human cDNA product secreted by mouse C127 cells (8a). In previous studies, the 200-kDa molecule was not detected in precipitates of culture medium from cells expressing the 1.6-kb cDNA, although this result may have been due to the relatively low levels of soluble CSF-1 obtained by [^{35}S]methionine labeling of these cells (19). However, the 200-kDa material was also not immunoprecipitated from culture supernatants of C127 mouse cells producing high levels of a soluble truncated product generated by the premature termination of the smaller CSF-1 precursor (4). At present, we cannot formally exclude the possibility that the 200-kDa material is a form of the 4-kb CSF-1 cDNA product that undergoes additional posttranslational modifications. However, insufficient [^{35}S]methionine was incorporated into the cell-associated 70-kDa CSF-1 molecule during pulse-chase analyses to account for recovery in both the soluble 43-kDa subunit and the secreted 200-kDa molecule, and a larger cell-associated product was not observed in the present study. The 200-kDa molecule may represent a cellular proteoglycan that binds the soluble CSF-1 homodimer in a manner analogous to that observed for heparin binding to fibroblast growth factors.

The physiological significance of the two human CSF-1 transcripts is presently unknown. Although the 4-kb mRNA is the predominant form detected in a variety of cell lines, the production of biologically active CSF-1 is correlated with the appearance of smaller transcripts in at least one inducible system (18). Additional transcripts formed by alternate splicing have been molecularly cloned and differ only in their 3′ untranslated sequences (7). Two mRNAs of the corresponding sizes are differentially expressed in various normal murine tissues (16), and the smaller transcript is the predominant one detected in pregnant-mouse uterus, in which CSF-1 is believed to have a role in placental development (15). However, the 2- and 4-kb CSF-1 cDNAs cloned from mouse L cells both encode the larger precursor and differ primarily in their 3′ untranslated sequences (7a). Thus, a murine CSF-1 product corresponding to the 256-amino-acid human protein has not yet been identified. The growth factors produced by the 1.6- and 4-kb human cDNAs were both biologically active in stimulating the proliferation of mononuclear phagocytes, and both transformed NIH-3T3 cells when coexpressed with the human c-*fms* gene. It is possible that the soluble products of the two cDNAs may differ with respect to their ability to elicit the full spectrum of pleiotropic responses induced by CSF-1. The fact that one mRNA codes for a rapidly secreted molecule whereas another produces primarily a plasma membrane-bound form suggests that CSF-1 can function both in cell-to-cell interactions and by paracrine or endocrine mechanisms.

ACKNOWLEDGMENTS

We thank Shawn Kramer, Lemuel Gorden III, and Virgil Holder for excellent technical assistance; Peggy Burdick for preparation of the manuscript; and Charles J. Sherr, in whose laboratory these studies were initiated, for his support and comments about the manuscript. We are grateful to the following individuals of Cetus Corporation: Martha Ladner for providing the pcDBCSF-4 plasmid, Michele Manos for communicating her results prior to publication, and Peter Ralph for his comments about the manuscript.

This work was supported by Public Health Service grants CA 38187 (to C. J. Sherr) and CA 21765 (Cancer Center CORE grant) from the National Institutes of Health and by the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

LITERATURE CITED

1. **Anderson, S. J., M. A. Gonda, C. W. Rettenmier, and C. J. Sherr.** 1984. Subcellular localization of glycoproteins encoded by the viral oncogene v-*fms*. J. Virol. **51**:730–741.
2. **Clark, S. C., and R. Kamen.** 1987. The human hematopoietic colony-stimulating factors. Science **236**:1229–1237.
3. **Coussens, L., C. Van Beveren, D. Smith, E. Chen, R. L. Mitchell, C. M. Isacke, I. M. Verma, and A. Ullrich.** 1986. Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. Nature (London) **320**:277–280.
4. **Heard, J. M., M. F. Roussel, C. W. Rettenmier, and C. J. Sherr.** 1987. Synthesis, post-translational processing, and autocrine transforming activity of a carboxyl terminal truncated form of colony stimulating factor-1. Oncogene Res. **1**:423–440.
5. **Kawasaki, E. S., M. B. Ladner, A. M. Wang, J. Van Arsdell, M. K. Warren, M. Y. Coyne, V. L. Schweickart, M. T. Lee, K. J. Wilson, A. Boosman, E. R. Stanley, P. Ralph, and D. F. Mark.** 1985. Molecular cloning of a complementary DNA encoding human macrophage-specific colony stimulating factor (CSF-1). Science **230**:291–296.
6. **Kuo, S. C., and J. O. Lampen.** 1974. Tunicamycin—an inhibitor of yeast glycoprotein synthesis. Biochem. Biophys. Res. Commun. **58**:287–295.
7. **Ladner, M. B., G. A. Martin, J. A. Noble, D. M. Nikoloff, R. Tal, E. S. Kawasaki, and T. J. White.** 1987. Human CSF-1: gene structure and alternative splicing of mRNA precursors. EMBO J. **6**:2693–2698.

7a. **Ladner, M. B., G. A. Martin, J. A. Noble, V. P. Wittman, M. K. Warren, M. McGrogan, and E. R. Stanley.** 1988. cDNA cloning and expression of murine macrophage colony-stimulating factor from L929 cells. Proc. Natl. Acad. Sci. USA **85**:6706–6710.

8. **Lee, M. T., and M. K. Warren.** 1987. CSF-1-induced resistance to viral infection in murine macrophages. J. Immunol. **138**:3019–3022.

8a. **Manos, M. M.** 1988. Expression and processing of a recombinant human macrophage colony-stimulating factor in mouse cells. Mol. Cell. Biol. **8**:5035–5039.

9. **Metcalf, D.** 1984. The haemopoietic colony stimulating factors. Elsevier/North-Holland Publishing Co. Amsterdam.

10. **Metcalf, D., and N. A. Nicola.** 1985. Synthesis by mouse peritoneal cells of G-CSF, the differentiation inducer for myeloid leukemia cells: stimulation by endotoxin, M-CSF, and multi-CSF. Leuk. Res. **9**:35–50.

11. **Morgan, C., J. W. Pollard, and E. R. Stanley.** 1987. Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.2F5. J. Cell. Physiol. **130**:420–427.

12. **Orci, L., M. Ravazzola, M.-J. Storch, R. G. W. Anderson, J.-D. Vassalli, and A. Perrelet.** 1987. Proteolytic maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory vesicles. Cell **49**:865–868.

13. **Pettenati, M. J., M. M. Le Beau, R. S. Lemons, E. A. Shima, E. S. Kawasaki, R. A. Larson, C. J. Sherr, M. O. Diaz, and J. D. Rowley.** 1987. Assignment of *CSF-1* to 5q33.1: evidence for clustering of genes regulating hematopoiesis and for their involvement in the deletion of the long arm of chromosome 5 in myeloid disorders. Proc. Natl. Acad. Sci. USA **84**:2970–2974.

14. **Plummer, T. H., Jr., J. H. Elder, S. Alexander, A. W. Phelan, and A. L. Tarentino.** 1984. Demonstration of peptide: *N*-glycosidase F activity in endo-β-*N*-acetylglucosaminidase F preparations. J. Biol. Chem. **259**:10700–10704.

15. **Pollard, J. W., A. Bartocci, R. Arceci, A. Orlofsky, M. B. Ladner, and E. R. Stanley.** 1987. Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature (London) **330**:484–486.

16. **Rajavashisth, T. B., R. Eng, R. K. Shadduck, A. Waheed, C. M. Ben-Avram, J. E. Shively, and A. J. Lusis.** 1987. Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. Proc. Natl. Acad. Sci. USA **84**:1157–1161.

17. **Ralph, P., and I. Nakoinz.** 1987. Stimulation of macrophage tumoricidal activity by the growth and differentiation factor CSF-1. Cell. Immunol. **105**:270–279.

18. **Ralph, P., M. K. Warren, M. T. Lee, J. Csejtey, J. F. Weaver, H. E. Broxmeyer, D. E. Williams, E. R. Stanley, and E. S. Kawasaki.** 1986. Inducible production of human macrophage growth factor, CSF-1. Blood **68**:633–639.

19. **Rettenmier, C. W., M. F. Roussel, R. A. Ashmun, P. Ralph, K. Price, and C. J. Sherr.** 1987. 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. Mol. Cell. Biol. **7**:2378–2387.

20. **Rettenmier, C. W., M. F. Roussel, C. O. Quinn, G. R. Kitchingman, A. T. Look, and C. J. Sherr.** 1985. Transmembrane orientation of glycoproteins encoded by the v-*fms* oncogene. Cell **40**:971–981.

21. **Rettenmier, C. W., R. Sacca, W. L. Furman, M. F. Roussel, J. T. Holt, A. W. Nienhuis, E. R. Stanley, and C. J. Sherr.** 1986. Expression of the human c-*fms* proto-oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. J. Clin. Invest. **77**:1740–1746.

22. **Roussel, M. F., T. J. Dull, C. W. Rettenmier, P. Ralph, A. Ullrich, and C. J. Sherr.** 1987. Transforming potential of the c-*fms* proto-oncogene (CSF-1 receptor). Nature (London) **325**:549–552.

23. **Roussel, M. F., C. W. Rettenmier, A. T. Look, and C. J. Sherr.** 1984. Cell surface expression of v-*fms*-coded glycoproteins is required for transformation. Mol. Cell. Biol. **4**:1999–2009.

24. **Sacca, R., E. R. Stanley, C. J. Sherr, and C. W. Rettenmier.** 1986. Specific binding of the mononuclear phagocyte colony-stimulating factor CSF-1 to the product of the v-*fms* oncogene. Proc. Natl. Acad. Sci. USA **83**:3331–3335.

25. **Sherr, C. J., C. W. Rettenmier, R. Sacca, M. F. Roussel, A. T. Look, and E. R. Stanley.** 1985. The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell **41**:665–676.

26. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–338.

27. **Stanley, E. R.** 1985. The macrophage colony stimulating factor, CSF-1. Methods Enzymol. **116**:564–587.

28. **Stanley, E. R., L. J. Guilbert, R. J. Tushinski, and S. H. Bartelmez.** 1983. CSF-1—a mononuclear phagocyte lineage-specific hemopoietic growth factor. J. Cell. Biochem. **21**:151–159.

29. **Stanley, E. R., and P. M. Heard.** 1977. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J. Biol. Chem. **252**:4305–4312.

30. **Tarentino, A. L., C. M. Gomez, and T. H. Plummer, Jr.** 1985. Deglycosylation of asparagine-linked glycans by peptide:*N*-glycosidase F. Biochemistry **24**:4665–4671.

31. **Umemoto, J., V. P. Bhavanandan, and E. A. Davidson.** 1977. Purification and properties of an endo-α-*N*-acetyl-D-galactosaminidase from *Diplococcus pneumoniae*. J. Biol. Chem. **252**:8609–8614.

32. **Warren, M. K., and P. Ralph.** 1986. Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. J. Immunol. **137**:2281–2285.

33. **Wong, G. G., P. A. Temple, A. C. Leary, J. S. Witek-Giannotti, Y.-C. Yang, A. B. Ciarletta, M. Chung, P. Murtha, R. Kriz, R. J. Kaufman, C. R. Ferenz, B. S. Sibley, K. J. Turner, R. M. Hewick, S. C. Clark, N. Yanai, H. Yokota, M. Yamada, M. Saito, K. Motoyoshi, and F. Takaku.** 1987. Human CSF-1: molecular cloning and expression of 4 kb cDNA encoding the human urinary protein. Science **235**:1504–1508.