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Biological Characterization of Uncleavable Plasma Membrane-Anchored Human Macrophage Colony-Stimulating Factor

Ping Deng, Ying-Lin Wang, Violette L. Shahbazian, and Paul K. Pattengale[1]
Department of Pathology, Childrens Hospital of Los Angeles and University of Southern California School of Medicine, Los Angeles, California 90027

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The cell-surface form of human macrophage colony-stimulating factor (CSF-1^{256}, M-CSFα) is a plasma membrane-anchored transmembrane protein from which the soluble CSF-1 is released by ectodomain proteolytic cleavage. We have previously generated two forms of cell surface CSF-1 which failed to undergo the cleavage by deleting residues 161–165 or residues 159–165 in the extracellular juxtamembrane region (1). To determine the biologic significance of the ectodomain cleavage, we compared the biosynthesis and biologic activities of uncleavable mutant CSF-1 forms with those of the cleavable wild-type (WT) CSF-1. We found that the uncleavable CSF-1 forms were able to accumulate on cell surface at about threefold higher level than the cleavable WT CSF-1 did. We further demonstrated that the uncleavable plasma membrane-anchored forms of CSF-1 were biologically active in mediating the proliferation of CSF-1-dependent cells as well as the intercellular adhesion between CSF-1 receptor-bearing cells and CSF-1 expressing cells. Furthermore, the adhesive activity of uncleavable CSF-1 forms was about twofold stronger than that of WT CSF-1, which indicated that the ectodomain cleavage system plays an important role in regulating the biologic activities of membrane-anchored CSF-1. **© 2000 Academic Press**

A class of growth factor precursors are expressed stably on cell surface as membrane-anchored growth factors (2). Some of these cell surface growth factors have been shown to bind to the target receptors on adjacent cells and then mediate intercellular adhesion and signal transduction (3–7). The extracellular domains of such cell surface growth factors are released as soluble growth factors by a regulated proteolytic cleavage process (2). This ectodomain cleavage process is also involved in the release of other cell surface proteins such as ectoenzymes, growth factor receptors, cell adhesion molecules, and β-amyloid precursor protein that has been implicated in the pathogenesis of Alzheimer's disease (2, 8–12).

CSF-1 is a disulfide-linked homodimeric glycoprotein that stimulates proliferation, differentiation, and survival of monocytes, macrophages, and their early bone-marrow progenitor lineage (13, 14). CSF-1 also enhance effector functions of mature mononuclear phagocytes and regulates release of other cytokines and inflammatory mediators (13, 14). CSF-1 has also been associated with regulation of placenta and bone development (15–17). More recently, it has been implicated in the pathogenesis of a variety of diseases such as atherosclerosis (18–20), preeclampsia (21), and human adenocarcinomas of ovary, endometrium, and breast (22–24). The diverse biological effects of CSF-1 are mediated by its binding to the CSF-1 receptor (CSF-1R), a ligand-activated protein tyrosine-kinase receptor that is identical to the c-*fms* protooncogene product (9, 13). CSF-1^{256} one of the three CSF-1 isoforms, is stably expressed on the cell surface as a plasma membrane-anchored growth factor (25). Cell surface CSF-1^{256} supports the formation of macrophage colonies in direct contact with CSF-1-expressing fibroblasts which are killed by chemical fixation (27). The extracellular domain of CSF-1^{256} undergoes ectodomain cleavage to yield biologically active soluble growth factor which is able to stimulate the proliferation of adjacent CSF-1 receptor (CSF-1R)-bearing cells (25, 26). This process is stimulated by protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA) (25, 26). In previous studies, we reported that the native conformation of the CSF-1^{256} growth factor

Abbreviations used: CSF-1 or M-CSF, macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; WT, wild-type; TM, transmembrane domain.

[1] To whom correspondence should be addressed at Department of Pathology, MS 103, Childrens Hospital of Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027. Fax: (323) 666-0489. E-mail: pattenga@hsc.usc.edu.

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domain and CSF-1^{256} dimerization are not required for the cleavage, (1) and that the structural requirements for both the cleavage efficiency and cleavage site selection are mainly determined by the small extracellular juxtamembrane region of CSF-1^{256} (1, 28).

Direct cell-cell interactions are critical events in embryonic development, morphogenesis of tissue, and maintenance of the adult organism (29–31). These interactions are mainly mediated by cell adhesion molecules. Cell adhesion molecules assemble animal cells into tissues by their adhesive properties and also regulate cell growth and differentiation by transducing a signal directly or via intracellular molecules associated with their cytoplasmic domains (31, 32).

Certain membrane-anchored growth factors have been found to act as adhesion molecules (6, 33–36). Growth factors are generally derived from their precursors that mature through proteolytic cleavage within the cell. These precursors are biologically inactive and their existence is confined to the cytoplasmic compartments where processing of secretory proteins occurs. However, a class of growth factor precursors do not undergo proteolytic processing within the cells but are expressed stably on cell surface as plasma membrane-anchored growth factors (2). Some of such cell surface growth factors have been shown to bind to the target receptors on adjacent cells and initiate signal transduction (3, 4). Furthermore, a few membrane-anchored growth factors such as TGF-α and stem cell factor have been shown to mediate intercellular adhesion between the cells expressing the growth factors and the cells expressing the growth factor receptor (6, 33–36).

We have previously generated two mutant CSF-1 forms which are expressed on cell surface without undergoing the ectodomain cleavage (1). To further explore the biological function of cell surface CSF-1^{256} and to determine the biologic significance of the membrane-anchored CSF-1 form and the ectodomain cleavage system, we tested the ability of membrane-anchored CSF-1 in mediating the intercellular adhesion of CSF-1R-bearing cells to CSF-1-expressing cells, and in promoting the localized growth of the CSF-1R-bearing cells, and we compared the biosynthesis and biological activities of the uncleavable mutant forms with those of the cleavable wild-type (WT) CSF-1.

MATERIALS AND METHODS

Cell lines and cell culture. Mouse NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium at 5% CO_2 in a water-saturated atmosphere. The medium was supplemented with 10% fetal calf serum (FCS, GIBCO), 2 mM glutamine, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml). Transfection and selection of NIH 3T3 cells expressing WT CSF-1^{256}, CSF-1^{256}-Δ159–165, CSF-1^{256}-Δ161–165 have been described previously (1). BAC1.2F5 cells (37) were derived from Simian virus 40-immortalized spleen cells and grew as adherent macrophage-like cells that are completely dependent on CSF-1 for proliferation and survival in culture. They were grown in DMEM medium supplemented with 15% FCS and 25% L cell-conditioned medium as a source of CSF-1 (27). M-NSF-60 cells (38) are CSF-1 dependent murine myeloid leukemia cells derived from NFS-60 cells (39) by selected growth in IL-3 plus human recombinant M-CSF. M-NFS-60 cells grow as round, nonadherent cells. They were maintained in RPMI 1640 medium supplemented with 10% FCS and 25% L cell-conditioned medium as a source of CSF-1.

Northern blot analysis. RNA was extracted from NIH 3T3 cells using the acid-guanidinium-phenol-chloroform method (40). 20 μg of RNA of each sample was electrophoresed through a 1.1% agarose/formaldehyde gel in MOPS X1 buffer, transferred to nylon membrane (Bio-Rad) and cross-linked by a UV-stratalinker (Stratagene, Inc., La Jolla, CA). The membranes were first pre-hybridized at 65°C in prehybridization solution (1 mM EDTA, 0.5 M Na_2HPO_4, pH 7.2, 7% SDS) for 1 h, then hybridized at 65°C with the same buffer containing ^{32}P-labeled cDNA probes prepared with a random-primed labeling kit (Boehringer Mannheim) for 16 h. After 16 h of hybridization, membranes were washed at 65°C three times for 30 min in wash buffer (1 mM EDTA, 40 mM Na_2HPO_4, pH 7.2, 5% SDS). The cDNA probes used were human CSF-1 cDNA fragment encoding the extracellular domain of CSF-1^{256} and human β-actin (BRL Life Technologies, Inc). Blots were stripped each time between hybridization by washing two times, 20 min each, in a large volume of 0.1× SSC/0.5% SDS at 95°C so that one blot was used for all 2 cDNA probes. Blots were exposed to Hyperfilm-MP (Amersham) with intensifying screens at −80°C for 3–6 days depending on the intensity of the signals.

For the quantitative analyses, the hybridized membranes were exposed to a molecular imaging screen, and the signals were quantitated by the Phosphor Analyst/PC (Bio-Rad). The quantity of CSF-1 expression was normalized to β-actin from the same blot to normalize the amount of RNA level. The data were analyzed and graphed by Sigma Plot 5.0.

Metabolic labeling, cell surface radioiodination, and immunoprecipitation. Stably transfected NIH 3T3 cells grown in 100 mm tissue culture plates were radioiodinated with ^{125}I (Amersham Corp., Arlington Heights, IL) and lactoperoxidase (Sigma Chemical Co., St. Louis, MO) as described previously (41). The cells were then harvested by detergent lysis for immunoprecipitation. The immunoprecipitation of human CSF-1 molecules was performed with the rat YYG106 monoclonal antibody (42). The controls for nonspecific precipitation were performed using an isotype-matched rat myeloma protein. Immunoprecipitation was carried out with Protein A-Sepharose (Pharmacia, Piscataway, NJ) precoated with a goat anti-rat IgG (Cappel) as the immunoadsorbant. The samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) under reducing or non-reducing conditions. The labeled proteins were detected by autoradiography, and quantified by densitometer. The apparent molecular weights of the labeled proteins were determined by comparison with the mobility of protein molecular weight standards.

Colony formation assay. NIH 3T3 cells with or without introduced human CSF-1 cDNA constructs, were grown to confluence on six well plates. The plates were fixed in 2% glutaraldehyde. M-NFS-60 cells or BAC1.2F5 cells, 1×10^6 cells per well, were added into the 6-well plates. After incubation at 37°C for 6 h in a humidified atmosphere with 5% CO_2 in air, the nonadherent cells were removed by gentle washing of cell layers with PBS. Then, cell layers were washed and replenished with fresh medium at 2 day intervals. After coculture for 7 to 14 days, colonies of adherent cells on the monolayers were examined and photographed under phase-contrast microscopy. Macrophage colony formation was examined and photographed against the background of the fixed monolayers. Macrophage colony were confirmed by staining either with hematoxylin and eosin or with α-naphthyl butyrate esterase and a light green S counterstain.

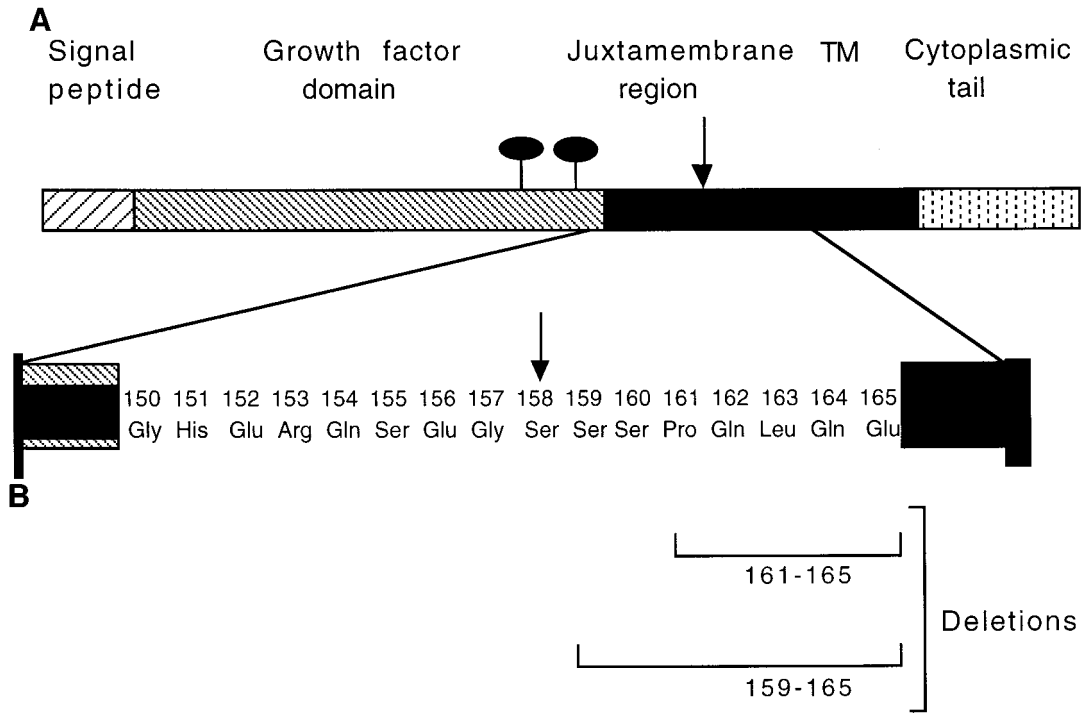

FIG. 1. Schematic representation of the human CSF-1^{256} proteins. (A) Domain structure and juxtamembrane sequence of human CSF-1^{256}. Amino acids are numbered from the aminoterminus of the soluble growth factor. Canonical sites for addition of N-linked glycosylation are indicated by closed ovals. The proteolytic cleavage site is indicated by an arrow. TM stands for transmembrane domain. (B) Deletion mutations introduced at or near the cleavage site of CSF-1^{256}.

Butyrate esterase is a cytochemical marker for mononuclear phagocytes, which show a reddish-brown, granular pattern of cytoplasmic staining (43). Antibody neutralization experiments were performed to demonstrate that macrophage colony formation is specifically dependent on CSF-1. Briefly, six well plates containing monolayers of vector-transfected NIH 3T3 cells, NIH 3T3 cells expressing WT CSF-1^{256}, CSF-1^{256}-Δ161–165, CSF-1^{256}-Δ159–165 were fixed as described above, and then incubated for 1 h at 37°C in 100 μl of PBS with either normal rabbit serum as a control or a rabbit neutralizing antiserum (44) to CSF-1 at a dilution of 1:50. This antibody at a dilution of 1:50 was found to block the colony formation of BAC1.2F5 cells on the fixed monolayer of NIH 3T3 cells expressing WT CSF-1^{256} (27). BAC1.2F5 cells were then added, and the following procedures were the same as described above.

Cell–cell adhesion assay. Monolayers of vector-transfected NIH 3T3 cells or NIH 3T3 cells expressing CSF-1^{256} were established in 24 well plates, and then incubated with 0.5 ml of M-NFS-60 cell suspension medium (2×10^6 cells/ml) at 37°C in a water-saturated atmosphere with 5% CO_2 in air. After 6 h of coculture, nonadherent cells were removed by gentle washing of cell layers with PBS. Adherent cells on the cell monolayers were observed and photographed under phase-contrast microscopy.

For the quantitative cell–cell adhesion assay, M-NFS-60 cells were pulse-labeled with 5 μCi/ml of [^{3}H]-thymidine for 4 h, washed three times with PBS, and then adjusted to 2×10^6 cells/ml in medium. 1×10^6 of [^{3}H]-labeled cells were added to confluent NIH 3T3 cell monolayers established on 24 well plates, and incubated at 37°C for 6 h in a humidified atmosphere with 5% CO_2 in air. Then the nonadherent cells were removed by gentle washing of cell layers with PBS. Adherent cells on the cell monolayers were harvested by trypsinization with the stromal cells, and the radioactivity of the collected cells were measured by a liquid scintillation counter.

RESULTS

The Uncleavable CSF-1 Forms Were Able to Accumulate on Cell Surface at a Higher Level than the Cleavable WT CSF-1 Did

The CSF-1^{256} precursor is composed of an aminoterminal signal peptide, a growth factor domain, a small juxtamembrane region, and a transmembrane domain (TM) followed by a short cytoplasmic tail (Fig. 1A). WT CSF-1^{256} is expressed as cell surface transmembrane proteins that undergo ectodomain cleavage to release a soluble growth factor (25). Deletion of region 159–165 or region 161–165 (Fig. 1B) abolished the cleavage, but still allowed for their efficient cell surface expression (1). Here, we asked whether the uncleavable forms accumulated at a higher level than the cleavable WT CSF-1 did or they can be degraded faster to keep same level of expression. To address this question, several transfectants of uncleavable CSF-1 forms were screened quantitatively by Northern blot to identify transfectants in which the level of mRNA expression of uncleavable CSF-1 forms was similar to that of WT CSF-1. It was found that the transfectant 10 of CSF-1^{256}-Δ161–165 (Fig. 2) and the transfectant 4 of CSF-1^{256}-Δ159–165 (data not shown) have mRNA expression levels similar to that of cells expressing WT CSF-1.

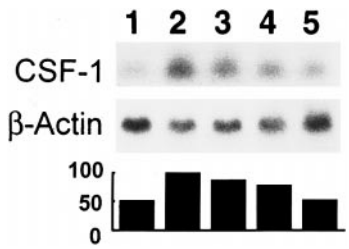

FIG. 2. Identification of trasfectant clones expressing mRNA level of CSF-1^{256}-Δ161–165 similar to that of WT CSF-1^{256}. The corresponding column on the graph shows the relative expression for each cell line (highest expression defined as 100). Total RNA was extracted from cell lines and subjected to Northern analysis with ^{32}P-labeled CSF-1 cDNA. Blots were stripped and re-probed with β-Actin cDNA to normalize the loading of RNA. Lane 1, WT; 2, CSF-1^{256}-Δ161–165-#2; 3, CSF-1^{256}-Δ161–165-#5; 4, CSF-1^{256}-Δ161–165-#7; 5, CSF-1^{256}-Δ161–165-#10.

To determine whether abolishment of the cleavage lead to higher levels of cell surface expression of CSF-1, transfected NIH 3T3 fibroblasts with similar CSF-1 mRNA expression: the transfectant 10 of CSF-1^{256}-Δ161–165 and transfectant 4 of CSF-1^{256}-Δ159–165 were enzymatically radioiodinated. The cell monolayers were then lysed in detergent containing buffer for immunoprecipitation. WT CSF-1^{256} was expressed as a 68-kDa disulfide-linked dimeric glycoprotein and a 56-kDa membrane-associated heterodimeric intermediate of the 34-kDa subunit and 22-kDa subunit (Fig. 3A) (25). In contrast, CSF-1^{256}-Δ161–165 or CSF-1^{256}-Δ159–165 was expressed as cell surface homodimeric protein of about 67 kDa, and the membrane-associated heterodimeric intermediate was not detected (Fig. 3A). Furthermore, quantitative analysis of the cell surface expression level indicated that uncleavable CSF-1 can accumulate 3× fold higher than WT CSF-1 did. To compare the kinetics of WT and cell surface uncleavable CSF-1, NIH 3T3 cells expressing similar amount of WT CSF-1 mRNA or CSF-1^{256}-Δ161–165 (transfectant 10) and CSF-1^{256}-Δ159–165 (transfectant 4) were enzymatically radioiodinated, and incubated with medium in the presence or absence of 0.5 μM PMA. At indicated times, the medium was collected and the cell monolayers were lysed for immunoprecipitation. WT CSF-1^{256} of 68 kDa with two identical subunits undergo sequential cleavage to yield a 56-kDa membrane-associated heterodimeric intermediate and a final soluble 44-kDa dimeric growth factor (Fig. 3B) (25). The cleavage was accelerated by PMA (Fig. 3B) (26). In contrast, CSF-1^{256}-Δ161–165 (Fig. 3B) and CSF-1^{256}-Δ159–165 (data not shown) were expressed as cell surface homodimeric protein of about 67 kDa, but did not generate the membrane-associated heterodimeric intermediate or the final soluble form in the presence or absence of PMA (Fig. 3B), which is consistent with our previous finding by metabolic labeling (1). Furthermore, the conditioned medium from cell expressing uncleavable CSF-1 did not have increased amount CSF-1 activity compared with the conditioned medium from vector-transfected NIH 3T3 cells as measured by a ^{3}H proliferation assay (data not shown).

Uncleavable CSF-1 Forms Were Biologically Active to Stimulate the Proliferation of CSF-1-Dependent Cells

NIH 3T3 fibroblasts producing various CSF-1 forms were fixed with glutaraldehyde and tested for their ability to stimulate the proliferation and colony formation of murine macrophage cells. BAC1.2F5 cells added to the glutaraldehyde-fixed monolayers of vector-transfected NIH 3T3 cells did not proliferate to form colonies (Fig. 4A) (27). In marked contrast, BAC1.2F5 cells added to the glutaraldehyde-fixed monolayers of NIH 3T3 cells expressing WT CSF-1^{256} (Fig. 4B) and uncleavable CSF-1 forms of CSF-1^{256}-Δ161–165 (Fig. 4C) and CSF-1^{256}-Δ159–165 (data not shown) produced cell colonies. The proliferating cells were confirmed to be mononuclear phagocytes by positive cytoplasmic staining with butyrate esterase, a cytochemical marker for macrophages (data not shown). To demonstrate the specificity of the formation of macrophage colonies in this assay, parallel assays were performed in the presence of neutralizing rabbit antiserum raised against human CSF-1. Incubation of the fixed monolayers with a 1:50 dilution of the neutralizing antiserum completely prevented macrophage colony formation (data not shown) (27).

We also examined whether M-NFS-60 cells adhering to monolayers of NIH 3T3 cells expressing WT CSF-1^{256} or uncleavable CSF-1 forms were able to proliferate. M-NFS-60 cells were cocultivated with confluent monolayers of NIH 3T3 cells expressing WT CSF-1^{256} or uncleavable CSF-1 forms for 6 h, which was followed by

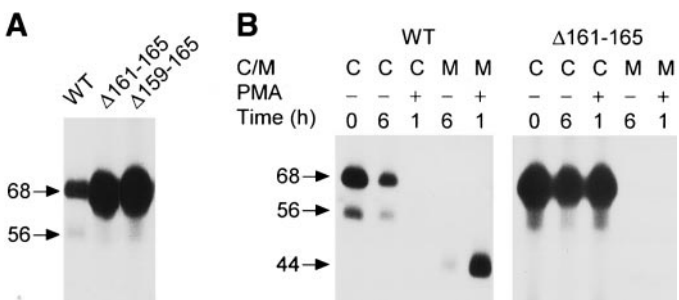

FIG. 3. Accumulation and kinetics of cell surface uncleavable CSF-1. (A) Transfected NIH 3T3 fibroblasts with similar CSF-1 mRNA expression were enzymatically radioiodinated. The cell monolayers were then lysed for immunoprecipitation. (B) NIH 3T3 cells stably transfected with either WT CSF-1^{256} or CSF-1^{256}-Δ161–165 were surface radioiodinated followed by incubation in either complete medium or in the same medium containing 0.5 μM PMA for the indicated intervals. Cell lysates and medium were immunoprecipitated with a monoclonal antibody to CSF-1 and analyzed by SDS–PAGE under nonreducing conditions and autoradiography. C, cell lysate; M, medium.

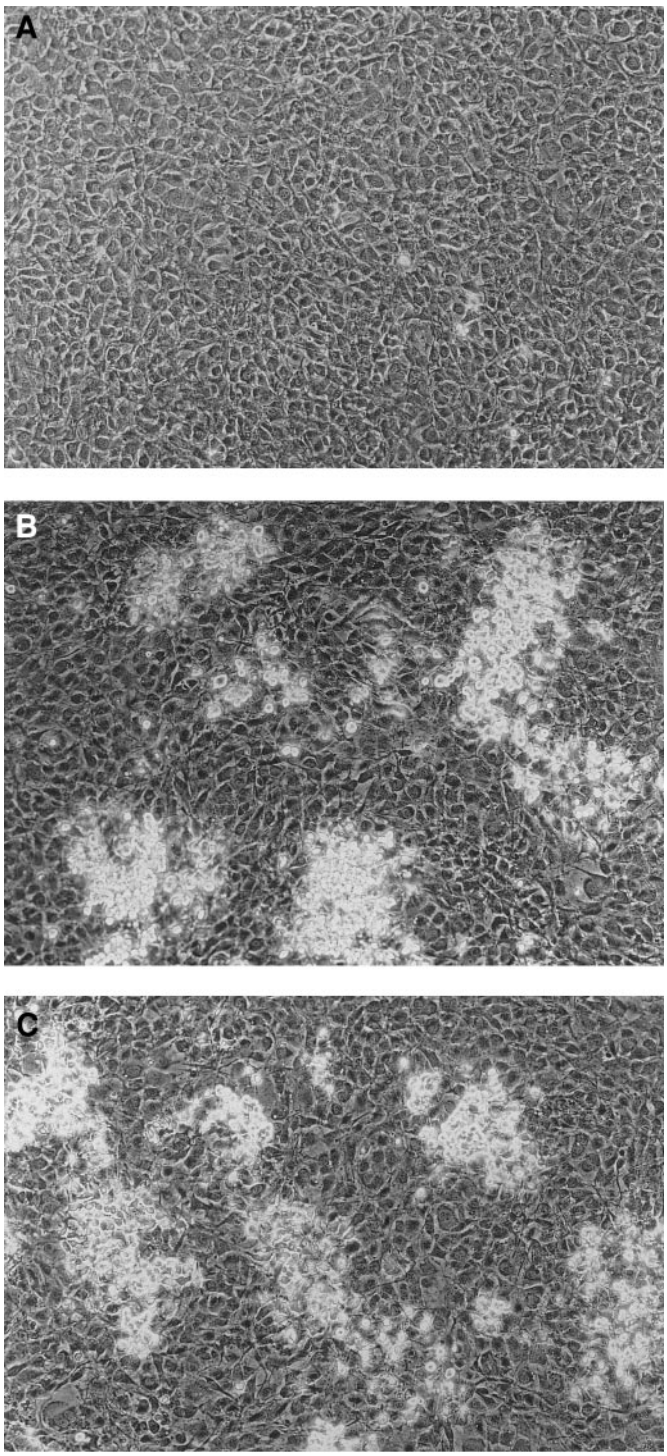

FIG. 4. Colony formation by BAC1.2F5 cells on glutaraldehyde-fixed monolayers of NIH 3T3 cells expressing human CSF-1 cDNA constructs. Bac1.2F5 cells were plated in medium lacking CSF-1 on glutaraldehyde-fixed monolayers of vector-transfected NIH 3T3 cells (A), NIH 3T3 cells transfected with CSF-1 cDNA forms of WT CSF-1^{256} (B) or CSF-1^{256}-Δ161–165 (C). Original magnification × 40.

removing the nonadherent cells and continuous cocultivation. It was found that M-NFS-60 cells formed colonies on the monolayers of NIH 3T3 cells expressing WT CSF-1^{256} (Fig. 5B), CSF-1^{256}-Δ161–165 (Fig. 5C), or CSF-1^{256}-Δ159–165 (data not shown), but not on the vector-transfected NIH 3T3 cells (Fig. 5A).

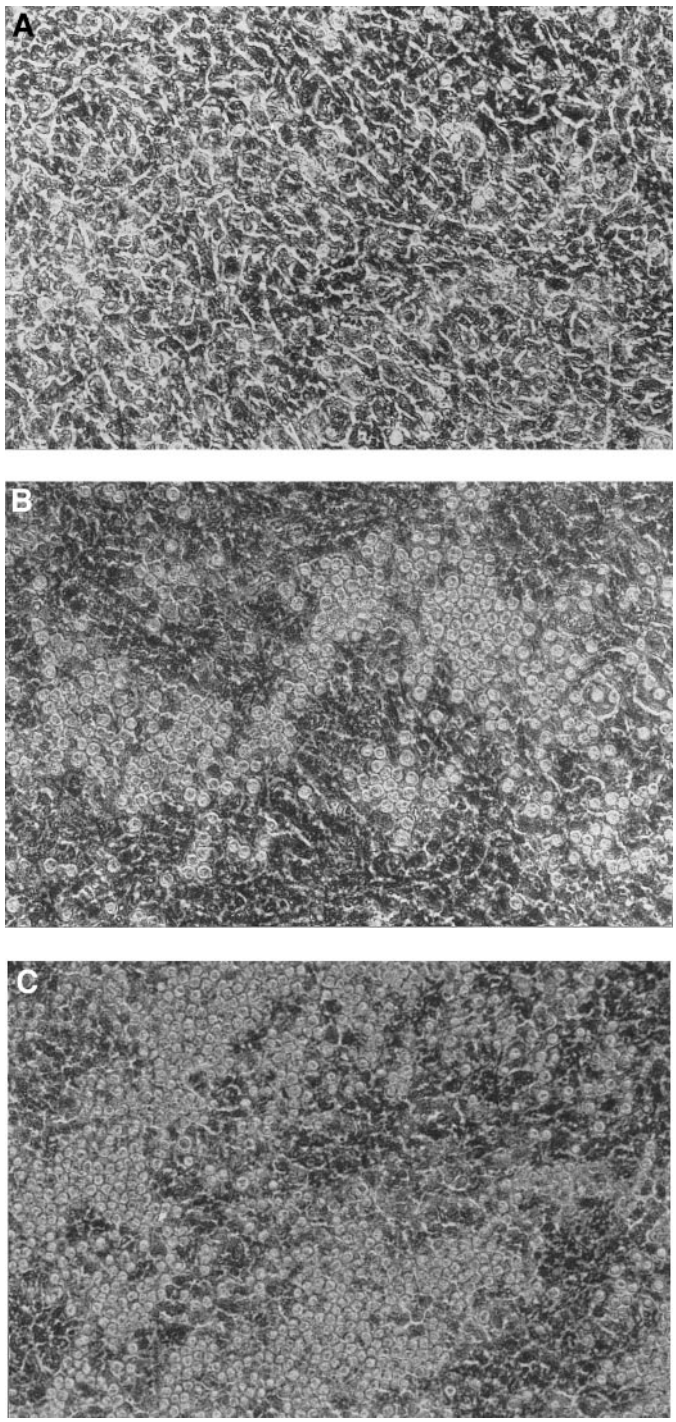

FIG. 5. Colony formation by M-NFS-60 on the monolayers of NIH 3T3 cells expressing human CSF-1 cDNA constructs. M-NFS-60 cells were plated in medium lacking CSF-1 on the monolayers of vector-transfected NIH 3T3 cells (A), NIH 3T3 cells transfected with CSF-1 cDNA forms of WT CSF-1^{256} (B) or CSF-1^{256}-Δ161–165 (C). Original magnification × 40.

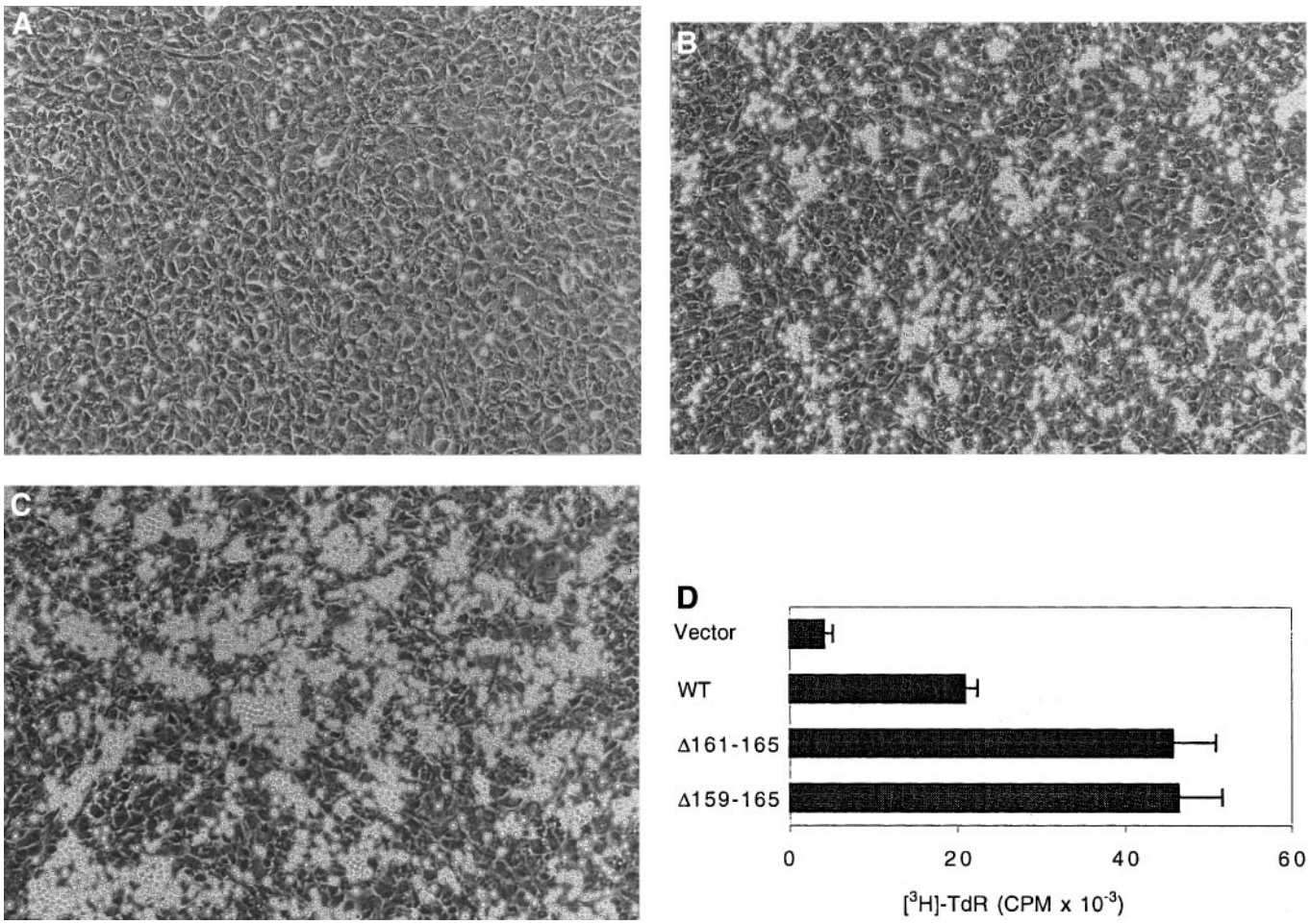

FIG. 6. Adhesion of M-NFS-60 to monolayers of NIH 3T3 cells expressing membrane-anchored CSF-1 forms. M-NFS-60 cells were incubated in 24-well microplates containing a preconstituted monolayer of vector-transfected NIH 3T3 cells (A), NIH 3T3 cells expressing WT CSF-1^{256} (B) or CSF-1^{256}-Δ161–165 (C). After 6 h incubation, nonadherent cells were removed by gentle washing. Adherent M-NFS-60 cells on the cell monolayers were photoed under phase-contrast microscopy with original magnification ×40. (D) Quantitative analysis of the adhesion of CSF-1R bearing cells to NIH 3T3 cells expressing membrane-anchored CSF-1. M-NFS-60 cells were pulse-labeled with [^{3}H]-thymidine, and were then added to cell monolayers of vector-transfected NIH 3T3 cells, NIH 3T3 cells expressing WT CSF-1^{256}, CSF-1^{256}-δ159–165 or CSF-1^{256}-Δ161–165. The radioactivity of the adherent cells was measured as described under Materials and Methods and graphed using SigmaPlot 5.0. Solid bars represent the mean ± SD of experiments performed in triplicate.

Uncleavable CSF-1 Forms Had Stronger Ability to Mediate the Adhesion between Cells Expressing Cell Surface CSF-1 and CSF-1R-Bearing Cells

CSF-1 dependent murine myeloid leukemia M-NFS-60 cells were coincubated with glutaraldehyde-fixed monolayers of vector-transfected NIH 3T3 cells or NIH 3T3 cells expressing CSF-1 constructs. As shown in Fig. 5, more M-NFS-60 cells were attached to NIH 3T3 cells producing uncleavable CSF-1^{256}-δ161–165 than WT CSF-1^{256} (Figs. 6B and 6C). To compare the adherance capacity of uncleavable CSF-1 with that of WT, a quantitative assay with [^{3}H]-thymidine-labeled cells was used. It was found that uncleavable CSF-1 led to about 2× fold increase in the ability in mediating the adhesion between cells expressing CSF-1 and cells expressing CSF-1R (Fig. 6D).

DISCUSSION

CSF-1^{256} is one of the membrane-anchored growth factors that is cleaved from the cell surface to yield biologically active soluble growth factors (25, 26, 44). We have previously generated two forms of cell surface CSF-1 which fail to undergo the cleavage by deleting residues 161–165 or residues 159–165 in the extracellular juxtamembrane region (1). In this study, these uncleavable CSF-1 forms were further analyzed together with the WT cleavable CSF-1 to determine the biologic activities of uncleavable CSF-1 forms and the biologic significance of the ectodomain cleavage system.

Our present study demonstrated that the cell-surface form of CSF-1 mediates the intercellular adhesion of CSF-1R-bearing cells to cells expressing

membrane-anchored CSF-1, which promotes localized proliferation of cells expressing CSF-1R. The cell-surface form of CSF-1 is expressed in bone marrow stromal cells (45), osteoblasts (46, 47) and cells of endometrial glands (48). Furthermore, the expression of membrane-anchored CSF-1 in osteoblast-like cells can be regulated by parathyroid hormone, tumor necrosis factor, and dexamethone treatment (49, 50). CSF-1R is expressed in monocytes, macrophages, osteoclasts, and their precursors (13, 14). CSF-1R is also expressed in cytotrophoblasts and cells of endometrial glands (13, 14). The biologic significance of the CSF-1-CSF-1R mediated cell–cell adhesion and proliferation is not well understood. It might play a critical role in developmental processes that require localized stimulation of the receptor-bearing target cells. It has been reported that membrane-anchored CSF-1 in osteoblasts is sufficient to support formation of multinucleated osteoclast-like cells (49). CSF-1-CSF-1R mediated cell–cell adhesion may also transmit certain signals in the opposite direction from CSF-1R-bearing cells to the cells expressing membrane-anchored CSF-1. It has been reported that intercellular adhesion between NFS-60 cells and MC3T3-G2/PA6 stromal cells induces granulocyte colony-stimulating factor production from the stromal cells (51). Whether NIH 3T3 cells can be induced to produce certain cytokines by cell–cell adhesion between M-NFS-60 cells and NIH 3T3 cells remains to be determined.

We demonstrate that both the WT and uncleavable mutant CSF-1 forms are able to stimulate the proliferation of CSF-1 receptor-bearing cells and to mediate the intercellular adhesion of these cells to CSF-1 expressing cells. Furthermore, the uncleavable CSF-1 forms are able to accumulate on the cell surface at a much higher level, and exert a much higher adhesive activity.

A previous report strongly suggested that cell surface CSF-1 supports the formation of macrophage colonies in direct contact with CSF-1-expressing fibroblasts which were killed by fixation with glutaraldehyde, formalin, or ethanol (27). However, this experiment did not exclude the possibility that the macrophage target cells which contain active proteases, may cleave the membrane-anchored CSF-1, and the resulting soluble CSF-1 initiates the signal transduction. The results in this study show that uncleavable CSF-1 is able to stimulate CSF-1R-bearing cells to proliferate, therefore demonstrate that the membrane-anchored CSF-1 itself is able to intiate the signal transduction of this growth factor, and the ectodomain cleavage of the membrane-anchored CSF-1 is not required to exert the growth-stimulating activity.

In this study, we demonstrated that uncleavable CSF-1 can accumulate on the cell surface at a much higher level than the WT CSF-1 did while both the uncleavable and WT CSF-1 forms have similar level of mRNA expression. Furthermore, uncleavable cell surface CSF-1 forms have stronger ability to mediate cell-cell adhesion. These results indicate that the protease responsible for the ectodomain cleavage of CSF-1^{256} can regulate the biologic activities of membrane-anchored CSF-1, suggesting an important role of the ectodomain cleavage system *in vivo*.

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