Structural Requirements for the Ectodomain Cleavage of Human Cell Surface Macrophage Colony-stimulating Factor

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One form of human macrophage colony-stimulating factor (CSF-1^{256}, M-CSF_{α}) is a member of a restricted set of cell surface transmembrane proteins, which is selected to undergo proteolytic ectodomain cleavage. To determine the substrate requirements for this cleavage, we have constructed a series of mutations in the cytoplasmic tail, transmembrane domain, and juxtamembrane region of CSF-1^{256} and stably expressed the mutated genes in NIH 3T3 cells. Our results demonstrate that membrane association of the CSF-1 precursor is required for cleavage of its growth factor ectodomain and furthermore that the juxtamembrane region Pro^{163}-Gln^{165} (PQLQE) (residues 161-165 of the ectodomain) is an essential determinant of cell surface CSF-1^{256} cleavage and that the cleavage site is partially sequence-specific. Furthermore, a mechanism of steric hindrance, which likely involves interference with protease accessibility, is postulated to explain the observed decreases in the cleavage efficiency in certain CSF-1 mutants. Finally, our results strongly suggest that the CSF-1 ectodomain is cleaved at or very near the cell surface by a membrane-associated proteolytic system.

For example, the failure to properly cleave β-APP may increase the production of amyloid β-proteins, which are invariably found in the senile plaques of Alzheimer's disease (Haass and Selkoe, 1993; Selkoe, 1994).

CSF-1^{256} (M-CSF_{α}) is one of the growth factors that is cleaved from the cell surface to yield biologically active soluble growth factors (Rettenmier et al., 1987; Halenbeck et al., 1988; Stein and Rettenmier, 1991). CSF-1 is a disulfide-linked dimeric glycoprotein regulating the growth, differentiation, and survival of cells of monocytic lineage (Stanley et al., 1983; Rettenmier and Sherr, 1989). It also plays an important role in the regulation of placental development and in bone osteoclast survival (Arceci et al., 1989; Wiktor-Jedrzejczak et al., 1990). Alternative splicing of the primary transcript yields multiple mRNAs encoding multiple forms of membrane-bound CSF-1 precursors (Kawasaki et al., 1985; Ladner et al., 1987). The largest human CSF-1 precursor of 554 amino acids (CSF-1^{544}, CSF-1pβ) is not expressed on the cell surface but is proteolytically cleaved within the cell and released into the extracellular compartment as both a soluble glycoprotein and a proteoglycan form with glycosaminoglycan chains (Rettenmier and Rousell, 1988; Price et al., 1992; Suzu et al., 1992). In contrast, the smallest CSF-1 precursor of 256 amino acids (CSF-1^{256}) is stably expressed on the cell surface (Rettenmier et al., 1987), and it is cell surface CSF-1^{256} that is biologically active in stimulating adjacent cells that express CSF-1 receptors (Stein et al., 1990). Furthermore, the growth factor domain of CSF-1^{256} is released from the cell surface into the extracellular medium by a regulated process of proteolytic cleavage. The rate of this cleavage is slow in unstimulated cells, but it is accelerated when cells are stimulated by certain agonists such as protein kinase C activators (Stein and Rettenmier, 1991).

Thus far, no protease(s) responsible for ectodomain cleavage of cell surface proteins has been identified. Furthermore, the structural determinants that specify such cleavage have not been clearly elucidated. We have therefore used the cell surface CSF-1^{256} cleavage system as a model to define the structural determinants that are required for cell surface protein ectodomain cleavage. In this study, we show that cell membrane association of CSF-1^{256} is required for its cleavage and that the CSF-1 juxtamembrane region (residues 150-165) is essential in determining the cleavage. In addition, a mechanism of steric hindrance, which likely involves interference with protease accessibility, might explain the observed decreases in the cleavage efficiency in certain CSF-1 mutants. Finally, we show that the cleavage of CSF-1^{256} occurs at or very near the cell surface and is mediated by a membrane-bound proteolytic enzyme system.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life
Ectodomain Cleavage of CSF-1

Technologies, Inc.), 2 mM glutamine, penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml).

Site-directed Mutagenesis—Human WT CSF-1-256 cDNA (Kawasaki et al., 1985) was subcloned into pBluescriptIISK(PD), a vector modified from pBluescriptIISK(--) (Stratagene) by deleting part of the polylinker region (674–719) to facilitate subcloning. A polymerase chain reaction mutagenesis technique was used for generation of each mutation (Steffan et al., 1989). Two internal mutagenizing primers and two external nonmutagenizing primers were used to generate two mutant DNA fragments having overlapping ends. These fragments were purified and used to serve as the template for a secondary polymerase chain reaction with the two external primers to produce the appropriate length of mutant DNA fragment. The secondary polymerase chain reaction products were subcloned back into pBluescriptIISK(PD) CSF-1-256. Only one internal mutagenizing primer containing the restriction enzyme site and one external nonmutagenizing primer were used if there was an appropriate restriction enzyme site near the site to be mutated. All sequences produced by polymerase chain reaction were confirmed by DNA sequencing using Sequenase (U.S. Biochemical Corp.).

Expression of CSF-1 Mutants in NIH 3T3 Cells—The mutant forms of CSF-1 cDNA in the pBluescriptIISK(PD) vectors were subcloned into an expression retrovector vector PSM, which is a replication-defective vector derived from the SM strain of feline sarcoma virus (Roussel et al., 1987). Restriction mapping was used to confirm the correct orientation of the inserts for expression. To generate stable transfectants, NIH 3T3 cells were cotransfected with circular plasmid DNA and pSV2neo by the CaPO4 coprecipitation technique. G418-resistant cell lines were screened by immunoprecipitation to identify stable cell lines that express mutant CSF-1 forms.

Metabolic Labeling—Cells expressing WT or mutant CSF-1 forms were metabolically labeled with [35S]methionine for 20 min and chased with complete medium containing 2% EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with 2% troponin treatment, cells were preincubated for 4 h in medium with tunicamycin (2 μg/ml) and labeled and chased in the continued presence of the drug.

Cell Surface Radioiodination—Cells grown in 100-mm tissue culture plates were washed once with phosphate-buffered saline and once with phosphate-buffered saline containing 5 mM glucose. The cells were incubated in 1.0 ml of phosphate-buffered saline containing 5 mM NaCl, 1.0 mM of carrier-free Na125I (Amersham Corp.), 75 μg of lactoperoxidase, and 8.8 μg of glucose oxidase (Sigma) for 5 min at 22°C and an additional 5 min at 4°C with occasional gentle agitation. After washing with cold phosphate-buffered saline twice, the cells were incubated at 37°C for variable amounts of time in complete medium with or without 0.5 μM PMA as indicated (Stein and Rettenmier, 1991). The medium was collected, and the cells were lysed in radioimmune precipitation lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with 2% aprotinin and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors for immunoprecipitation. For metabolic labeling under tunicamycin treatment, cells were preincubated for 4 h in medium with tunicamycin (2 μg/ml) and labeled and chased in the continued presence of the drug.

Cell Surface Radioiodination—Cells grown in 100-mm tissue culture plates were washed once with phosphate-buffered saline and once with phosphate-buffered saline containing 5 mM glucose. The cells were incubated in 1.0 ml of phosphate-buffered saline containing 5 mM NaCl, 1.0 mM of carrier-free Na125I (Amersham Corp.), 75 μg of lactoperoxidase, and 8.8 μg of glucose oxidase (Sigma) for 5 min at 22°C and an additional 5 min at 4°C with occasional gentle agitation. After washing with cold phosphate-buffered saline twice, the cells were incubated at 37°C for variable amounts of time in complete medium with or without 0.5 μM PMA as indicated (Stein and Rettenmier, 1991). The medium was collected, and the cells were lysed in radioimmune precipitation lysis buffer with 2% aprotinin and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors for immunoprecipitation. Immunoprecipitation—The primary antibody used was the YGG-106 rat monoclonal antibody (Stanley, 1985). Immunoprecipitation was carried out with protein A-Sepharose (Pharmacia Biotech Inc.) precoated with a goat anti-rat IgG (Cappel) as immunoadsorbant. The control for nonspecific precipitation was performed using an isotype-matched rat myeloma protein. Washed immune precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Anderson et al., 1984). The labeled proteins were detected by fluorography and quantitated with Betascope 603 (Betagen). Murine CSF-1 produced by parental NIH 3T3 cells was not detected by immunoprecipitation (Rettenmier et al., 1987). The apparent molecular weights of the labeled proteins were determined by comparison with the mobilities of protein molecular weight standards.

RESULTS

Ectodomain Cleavage of CSF-1-256 Requires Membrane Association—The CSF-1-256 precursor consists of an amino-terminal signal peptide, a growth factor domain, a small juxtamembrane region, and a transmembrane domain (TM) followed by a short cytoplasmic tail (Fig. 1A). CSF-1-256 is synthesized as a 68-kDa disulfide-linked dimeric glycoprotein whose two identical subunits undergo sequential cleavage to yield a 56-kDa membrane-associated heterodimeric intermediate and a final soluble 44-kDa dimeric growth factor. The cleavage can be accelerated by PMA (Fig. 1B) (Stein and Rettenmier, 1991). To address whether the cytoplasmic tail is required for the cleavage, NIH 3T3 cells were transfected with either WT CSF-1-256 or CSF-1-256-TC31 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 lysates; M, medium. Panel C, CSF-1-256-DTM16 was secreted into medium without cleavage. [35S]Methionine-labeled CSF-1-256 in the medium conditioned by NIH 3T3 cells expressing CSF-1-256-DTM16 with (lane 2) or without (lane 1) 0.5 μM PMA treatment for 3 h was incubated with antibody to CSF-1, and washed immune complexes were analyzed by fluorography after SDS-PAGE under nonreducing conditions. The apparent molecular masses of CSF-1 molecules are noted in kilodaltons (kDa) on the left.

To define the role of the transmembrane domain in the cleavage, a CSF-1 mutant with the deletion of 16 amino acids (residues 170–185) in the transmembrane domain (CSF-1-256, ΔTM16) was produced and stably expressed in NIH 3T3 cells. CSF-1-256,ΔTM16 was not expressed on the cell surface as demonstrated by cell surface radioiodination (data not shown). After metabolic labeling of cells expressing CSF-1-256,ΔTM16, full-length CSF-1-256,ΔTM16 with a molecular mass of 64 kDa was secreted into the medium without cleavage (Fig. 1C). Furthermore, the secretion of CSF-1-256,ΔTM16 was not stimulated by PMA (Fig. 1C). Taken together, these results demonstrate that membrane association of the CSF-1-256 precursor is required for the cleavage.

PQLQ (Residues 161–165) in the juxtamembrane Region Is an Essential Determinant of CSF-1-256 Ectodomain Cleavage—To define the role of the juxtamembrane region in the cleavage process, four deletion mutants spanning this region were constructed and stably expressed in NIH 3T3 cells. Dele-
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A

150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165
Gly His Glu Arg Gln Ser Glu Gly Ser Ser Ser Pro Glu Leu Gin Glu

B

| Cleavage | WT | Deletions | Substitutions |
|----------|----|----------|--------------|
| WT       | +++| Δ150-156 | Ala'150'156 |
| Δ150-156 | ++ | Δ150-156 | Asp'150'156 |
| Δ159-165 | –  | Δ159-165 | Lys'159'165 |
| Δ161-162 | +/–| Δ161-162 | Pro161Ala |
| Δ163-165 | +/–| Δ163-165 | Pro161Gly |
| Δ161     | ++ | Δ161     | Gin162Glu |
| Δ162     | ++ | Δ162     | Gin162Lys |
| Δ161-162 | +/–| Δ161-162 | Gin162Pro |

Fig. 2. Ectodomain cleavage of CSF-1256 forms with mutations in the juxtamembrane region. A, amino acid sequence of extracellular juxtamembrane region in CSF-1256. B, effects of mutations in the CSF-1256 juxtamembrane region on PMA-activated cleavage. NIH 3T3 cells stably transfected with either WT CSF-1256 or mutant CSF-1256 forms were metabolically labeled for 20 min and chased for 1 h in medium for maturation. The label was chased with fresh medium containing 0.5 μM PMA for 3 h. Medium and cell lysates were incubated with anti-CSF-1 antibody, and washed immunoprecipitates were subjected to SDS-PAGE under nonreducing conditions followed by quantitative fluorography. The cleavage efficiency of CSF-1256 is represented by symbols: the portion cleaved was <2% (+/+), 2–10% (+/–), 10–30% (+), 30–70% (+/+), 70–90% (+/+), >90% (+/++/+).

Steric Hindrance of Protease Accessibility Reduces Cleavage Efficiency—Deletion of region 150–156 in CSF-1256 severely impaired cleavage (Figs. 2 and 3). This deletion is located N-terminal to the cleavage site as well as the essential PQLQE region (residues 161–165) for cleavage and that region 150–160 contributes to the efficiency of the cleavage.

To further characterize the PQLQE region, a series of deletions were constructed and stably expressed in NIH 3T3 cells (Fig. 2). Deletion of region 161–162 (CSF-1256Δ161-162) or region 163–165 (CSF-1256Δ163-165) dramatically reduced the rate of cleavage (Figs. 2 and 3). Furthermore, deletion of even one amino acid, Pro161 or Gin162, also substantially reduced the cleavage efficiency (Fig. 2).

A series of substitution mutants were also constructed and stably expressed in NIH 3T3 cells to test the effects of amino acids with different side chain size and charge and amino acids that might change the normal secondary structure of the juxtamembrane region in CSF-1256 protein (Fig. 2). Pro161 was mutated to Ala, Gly, or Ser. Each of these three substitutions interfered with the cleavage process. Of the three mutations, Pro161Ser reduced the rate of cleavage the most as compared with that of WT (Figs. 2 and 3). Gin162 was mutated to Glu, Lys, or Pro. Each of the three mutant CSF-1256 forms was cleaved as efficiently as WT (Fig. 2). Substitutions, including change of Leu163 to Pro, Gin164 to Glu, Gin164 to Lys, and Glu165 to Gin, did not interfere with cleavage (Fig. 2). However, substitution of Leu163Gln164 with Ile-Pro substantially reduced the rate of cleavage (Fig. 2). These results are summarized in Fig. 2 and show that the PQLQE region (residues 161–165) is essential for cleavage, that the length of this PQLQE region is critical, and that the amino acid sequence requirement in this PQLQE region is partially specific.

The cleavage site (residues 157–159) in the juxtamembrane region is partially sequence-specific. Results from a previous study suggest that cell surface CSF-1256 is cleaved at or near Ser158 (Halenbeck et al., 1988). A deletion encompassing the cleavage site (CSF-1256Δ156–160) substantially reduced the rate of cleavage but did not completely abolish the cleavage, suggesting that the cleavage is partially sequence-specific in this region. To test this point further, we changed Ser158 to amino acids with different side chain size and charge, including CSF-1256Δ156–160 Asp158, CSF-1256Δ156–160 Lys158, CSF-1256Δ156–160 Gln158, CSF-1256Δ156–160 Pro158, and CSF-1256Δ156–160 Glu158. All of these amino acid replacements reduced the cleavage efficiency (Fig. 2). Asp substitution reduced the rate of cleavage to about 20% of the rate of WT. Leu and Lys substitutions reduced the rate of cleavage to about 40% of the rate of WT, whereas Ala substitution had the least effect on cleavage. These studies therefore show that the cleavage site of CSF-1256 is partially sequence-specific.

The cleavage site of CSF-1256 is partially sequence-specific.
CSF-1<sup>256</sup> with tunicamycin treatment is similar to that without tunicamycin treatment. In the presence of tunicamycin, WT CSF-1<sup>256</sup> was synthesized as a 46-kDa homodimeric polypeptide, and its two subunits were cleaved slowly into 41-kDa membrane-bound heterodimeric intermediate and 37-kDa soluble CSF-1 (Fig. 4). The cleavage was activated by PMA (Fig. 4). Similarly, the cleavage rate of CSF-1<sup>256</sup> Pro161Ser was not changed by tunicamycin treatment (Fig. 4). Furthermore, CSF-1<sup>256</sup>-161–165 was still not cleaved in the presence of tunicamycin, which further demonstrates the essential role of the PQLOE region in the cleavage (Fig. 4). In marked contrast, the cleavage rate of CSF-1<sup>256</sup>-150–156 was increased from 20% to more than 90% of the rate of WT by tunicamycin treatment (Fig. 4). Likewise, the cleavage rate of CSF-1<sup>256</sup>-156–160 was increased by tunicamycin treatment (data not shown). These results strongly suggest that the N-linked oligosaccharides in CSF-1 mutants with a shorter juxtamembrane region sterically interfere with the accessibility of the protease(s), therefore reducing the cleavage rate.

Effect of Calcium Ionophores on CSF-1<sup>256</sup> Cleavage and Subcellular Location of the Cleavage System—Calcium ionophores have been shown to activate the cleavage of certain cell surface proteins such as pro-TGF<sub>α</sub> (Pandella and Massagué, 1991). To test their role in CSF-1<sup>256</sup> cleavage, NIH 3T3 cells expressing CSF-1<sup>256</sup> were metabolically labeled and chased with medium containing calcium ionophores. In contrast to PMA, the calcium ionophores A23187 and ionomycin (500 nM, data not shown) did not stimulate the cleavage of CSF-1<sup>256</sup>.

Cell surface radioiodinated CSF-1<sup>256</sup> can be cleaved into soluble CSF-1 (Fig. 1), which demonstrates that cell surface CSF-1<sup>256</sup> can serve as the immediate precursor for cleavage. This raised the question of whether or not the cleavage occurs at or near the cell surface or in the lysosomal compartment, which is the cellular site of general membrane protein degradation. Cell surface detection of the heterodimeric intermediate of 56 kDa that contains one proteolyzed polypeptide chain (22 kDa) assembled through disulfide bonds to intact subunit (34 kDa) (Fig. 1) favors the first explanation. To further test this, the lysosomotropic agent chloroquine (Kornfeld and Mellman, 1989) was used. Chloroquine did not prevent either constitutive or PMA-induced cleavage of CSF-1<sup>256</sup> (Fig. 5). These studies suggest that the lysosomal compartment is not involved in the cleavage process.

We also asked whether CSF-1<sup>256</sup> is cleaved before it is transported to the cell surface. To answer this question, we used brefeldin A, an agent that blocks Golgi apparatus by inducing a resorption of the Golgi apparatus into the endoplasmic reticulum, and fusion of the trans-Golgi network with the endosomal system (Klausner et al., 1992). Normal CSF-1<sup>256</sup> maturation was blocked under brefeldin A treatment, and an underglycosylated CSF-1<sup>256</sup> with a smaller molecular mass (62 kDa) was detected (Fig. 6A). Both constitutive and PMA-induced cleavage of CSF-1<sup>256</sup> were blocked if brefeldin A was present from the start of labeling (Fig. 6A). In contrast, brefeldin A did not prevent the cleavage if CSF-1<sup>256</sup> had been chased to the cell surface (Fig. 6B). Resistance of CSF-1<sup>256</sup> to ectodomain cleavage was not due to underglycosylation of this protein by brefeldin A treatment, since nonglycosylated CSF-1<sup>256</sup> can be cleaved (Fig. 4). Thus, brefeldin A prevents the cleavage by blocking the transport of CSF-1<sup>256</sup> to the cell surface, suggesting that the cleavage occurs in subcellular sites post Golgi compartment. Collectively, these results indicate that the cleavage system operates at or near the plasma membrane and outside the Golgi or lysosomal compartments.

**DISCUSSION**

We have used CSF-1<sup>256</sup> as a model system for the ectodomain cleavage of cell surface transmembrane proteins. Previous work has shown that CSF-1<sup>256</sup> is expressed on the cell surface, where it contacts and activates CSF-1 receptors on adjacent cells (Rettenmier et al., 1987; Stein et al., 1990). The growth factor domain is released very slowly by proteolytic cleavage, and this process is activated by phorbol ester (Rettenmier et al., 1987; Stein and Rettenmier, 1991). The present results identify and characterize the structural determinants required for the release of the CSF-1<sup>256</sup> extracellular growth factor domain and demonstrate that the proteolytic cleavage system operates at or very near the cell surface.

The PQLOE region (residues 161–165) is the essential determinant of the cleavage, and the region 150–160 encompassing the cleavage site contributes to the cleavage efficiency. The coding regions for all of the three CSF-1 cDNA forms isolated so far share a common amino-terminal domain of 149 amino acids that contains the entire CSF-1 cytokine module, forming a globular structure (Bazan, 1991; Pandita et al., 1992). In addition, CSF-1<sup>256</sup> is cleaved at or near Ser<sup>159</sup> (Halenbeck et al., 1988). Furthermore, native conformation including dimer for-
Ectodomain Cleavage of CSF-1

![Fig. 6. Effect of brefeldin A on the biosynthesis and cleavage of CSF-1256](image)

**Fig. 6.** Effect of brefeldin A on the biosynthesis and cleavage of CSF-1256. A, cells were preincubated in medium with brefeldin A (10 μg/ml) and then metabolically labeled for 20 min and chased for 1 h for maturation. The medium was replaced with fresh medium to continue the chase for 2 h. Where indicated, PMA (0.5 μM) was added 1 h before the end of chase. Brefeldin A was included in both the labeling and chase medium. B, cells were metabolically labeled for 20 min and chased for 1 h in the absence of brefeldin A (20 μg/ml). The chase continued in fresh medium containing brefeldin A in the absence or presence of 0.5 μM PMA. 44-kDa CSF-1 in medium without PMA was detected by fluorography with longer exposure time (not shown).

**Fig. 7. A model for CSF-1256 ectodomain cleavage.** The growth factor domains are represented by large open ovals. N-Linked oligosaccharides are indicated by small filled ovals. The juxtamembrane region (residues 150–165) is depicted as follows: 150–156 (stippled boxes), 157–160 (solid lines), and 161–165 (hatched boxes). The transmembrane domains are shown as filled boxes, and the cytoplasmic tails are indicated by open boxes. The putative membrane-bound protease(s) is represented by E.

Interference by which the extracellular domains of certain cell surface proteins such as low density lipoprotein receptor are rapidly released from the cell surface by the proteolytic system in mutant Chinese hamster ovary cells with a defect in protein O-glycosylation (Kozarsky et al., 1988; Reddy et al., 1989). By inference from our findings, it is possible that the stalk-associated O-linked oligosaccharides in these cell surface proteins do not directly protect them from the ectodomain cleavage but interfere with the cleavage by steric hindrance of protease accessibility.

Existence of critical juxtamembrane determinants might be a general mechanism for cell surface transmembrane protein cleavage. Mutations in the cleavage site or its surrounding region inhibit or abolish the cleavage of certain cell surface proteins such as TGFα (Wong et al., 1989), tumor necrosis factor α (TNFα) (Perez et al., 1990; Decoster et al., 1995), and TNF receptor (Gullberg et al., 1992; Brakebusch et al., 1994). Deletion and substitution mutants in the juxtamembrane region of β-APP, including a deletion of amino acids 5–24 (numbered from the start of Aβ) within Aβ as well as a deletion of amino acids 11–28, still allow for efficient cleavage (Sisodia, 1992; Sahasrabudhe et al., 1992; Maruyama et al., 1991). By inference from our results, the explanation may be that β-APP has a very long exposed juxtamembrane stalk region. After 18 or 20 amino acid deletions in this region, the mutant β-APPs may still have the appropriate length of the exposed stalk region, which contains the enzyme binding site and cleavage site and allows for the sterer accessibility of the protease as well. Consistent with our assumption, it has been documented that more extensive deletions of juxtamembrane region in a modified β-APP (APP770Δ35) abolish the cleavage (Sisodia et al., 1990).

Significant differences might exist between some components of the proteolytic system responsible for the cleavage of various groups of proteins. The cytoplasmic C-terminal valine of pro-TGFα is essential for the cleavage (Bosenberg et al., 1992), but the cytoplasmic domains of cell surface CSF-1256, β-APP (Hung and Selkoe, 1994), and TNF receptor (Brakebusch et al., 1994) are not. In addition, calcium ionophores activate the cleavage of pro-TGFα (Pandiella and Massagué, 1991), KL-1, and KL-2 (Huang et al., 1992) but not CSF-1256 and ACE (Ramchandran et al., 1994). Finally, protease inhibitors blocking the cleavage of pro-TGFα, KL-1, and KL-2 (Pandiella et al., 1992) do not inhibit the cleavage of either CSF-1256 (data not shown) or ACE (Ramchandran et al., 1994). Recently, it has been reported that TNFα protease inhibitor does not affect the proteolytic processing of CSF-1254 while abolishing the ectodomain cleavage of the interleukin-6 receptor and the p60 TNF receptor (Mühlberg et al., 1995). It would have been

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The results in this study strongly suggest that the extracellular growth factor domain of CSF-1 \textsuperscript{256} is cleaved and released into the extracellular compartment by a membrane-associated proteolytic enzyme(s). First, a mutant CSF-1 \textsuperscript{256} precursor lacking a functional membrane-anchoring domain is released intact without being cleaved, although it still contains the essential cleavage determinant PQLOE, the authentic cleavage site, and the appropriate length of juxtamembrane stalk region. Second, direct phosphorylation of the cytoplasmic domain of CSF-1 \textsuperscript{256} does not interfere with the cleavage. Therefore, it is possible that the protease(s) involved is a transmembrane protein(s), the cytoplasmic domain of which directly or indirectly interacts with protein kinase C.

We have synthesized the results of this study in the proposed model for CSF-1 \textsuperscript{256} ectodomain cleavage (Fig. 7). Homodimeric CSF-1 \textsuperscript{256} of 68 kDa is expressed on the cell surface with the 16-amino acid juxtamembrane region (residues 150–165) being long enough to allow for the accessibility of the putative membrane-associated proteolytic enzyme(s) to the CSF-1 \textsuperscript{256} substrate. One domain of the proteolytic enzyme(s) recognizes and binds to the essential determinant PQLOE (residues 161–165). This binding leads to conformational changes of the enzyme(s) and brings the cleaving domain of the enzyme(s) close to the cleavage site (residues 157–159) suitable for cleavage and located at a specified distance of approximately 7–9 amino acids from the plasma membrane. Subsequently, the 44-kDa extracellular growth factor domain of CSF-1 \textsuperscript{256} is cleaved and released into the extracellular compartment. Since the two identical subunits are not always cleaved at the same time, a membrane-bound 56-kDa heterodimeric intermediate is often detectable on the cell surface.

Mutations that change the essential binding determinant of the protease(s), mutations that change the amino acid sequence of the cleavage site, or mutations that sterically interfere with the enzyme accessibility can reduce or abolish the cleavage.

Acknowledgments—We thank Kirston Koths and Ernest Kawasaki of Chiron Corporation for the CSF-1 \textsuperscript{256} cDNA, helpful discussions, and critical reading of the manuscript. We are grateful to Emerich Stein for the construction of two deletion mutants (1150–156 and 1156–160), to Julius Peters and Cheng-Ming Chuong for reading the manuscript and providing helpful comments, and to Ying-Lin Wang for assistance in the preparation of the manuscript. We also thank E. Richard Stanley for providing CSF-1 antibody.

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