Multiple Factors Determine the Selection of the Ectodomain Cleavage Site of Human Cell Surface Macrophage Colony-Stimulating Factor†

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Received July 17, 1998; Revised Manuscript Received September 22, 1998

ABSTRACT: Human cell surface macrophage colony-stimulating factor (CSF-1^{256}, M-CSFα) is converted to a soluble growth factor by a regulated proteolytic cleavage process at amino acid residues 157−159. We have previously shown that multiple factors specified by the juxtamembrane region determine the cleavage efficiency [Deng, P., Rettenmier, C. W., and Pattengale, P. K. (1996) *J. Biol. Chem. 271*, 16338−16343]. In the present paper, we studied the effect of various deletion, insertion, and substitution mutations at or near the cleavage site on both the number and size of cleaved CSF-1^{256} products to identify the mechanisms by which the cleavage sites are selected. Deletion of regions 161−162 or 163−165, C-terminal to the cleavage site, as well as deletion of region 150−156, N-terminal to the cleavage site, each yielded a single cleavage product that was smaller than that derived from the wild type (WT). In these experiments cleavage apparently occurred at a specific distance from the transmembrane domain. Insertion of three additional residues between the normal cleavage site and the transmembrane domain yielded one major product that was the same size as the processed form of WT CSF-1^{256}. In this case the selection of the cleavage site was apparently determined by the amino acid sequence of the juxtamembrane region rather than by the distance from the transmembrane domain. Other amino acid substitutions at the cleavage site caused changes in cleavage site selection, providing additional evidence for the role of amino acid sequence in cleavage site selection. Finally, a comparison of cleavage site selection in the presence and absence of tunicamycin treatment showed that N-glycosylation of certain mutant forms of CSF-1^{256} sterically interfered with protease accessibility, which in turn had an effect on the selection of the site used for cleavage. Taken together, these results indicate that cleavage site selection is determined by the amino acid sequence of the juxtamembrane region, the distance of the site from the transmembrane domain, and steric accessibility of the protease.

The extracellular domains of a variety of cell surface proteins are released as soluble and bioactive fragments by a regulated proteolytic cleavage process. This ectodomain cleavage process is involved in the regulation of the biological function of cell surface growth factors, ectoenzymes, growth factor receptors, and cell adhesion molecules (*1*−*5*). It is also involved in the processing of β-amyloid precursor protein (β-APP)[1] that has been implicated in the pathogenesis of Alzheimer's disease (*6*). Processing of such cell surface proteins by membrane proteases is becoming an intensively studied aspect of a variety of diseases (*5*).

CSF-1^{256} (M-CSFα), one of the three CSF-1 isoforms, undergoes ectodomain cleavage to yield biologically active soluble growth factors (*7*, *8*). CSF-1 regulates the growth, differentiation, and survival of cells of monocytic lineage (*7*, *9*). It also regulates placental development and bone osteoclast survival (*10*−*12*). Furthermore, CSF-1 has been implicated in the pathogenesis of a variety of diseases including atherosclerosis (*13*−*15*) and preeclampsia (*16*), as well as cancers of the breast and ovary (*17*−*19*). CSF-1^{256} is stably expressed on the cell surface as a membrane-anchored growth factor (*20*). Cell surface CSF-1^{256} supports the formation of macrophage colonies in direct contact with CSF-1-expressing fibroblasts, which were killed by chemical fixation (*21*). CSF-1^{256} expressed on the cell surface of tumor cells can induce macrophages to recognize and kill the tumor cells bearing cell surface CSF-1 (*22*). Furthermore, cell surface CSF-1^{256} mediates intercellular adhesion between CSF-1 receptor-bearing cells and CSF-1-expressing cells, and the biologic activities of membrane-anchored CSF-1 are regulated by the ectodomain cleavage system.[2]

Despite the broad biological significance of these cell surface cleavage mechanisms, little is known regarding the nature of the proteases responsible for the ectodomain

† This research was supported by a CHLA career development fellowship (to P.D.) and by the Las Madrinas Society of Childrens Hospital Los Angeles.

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[1] Abbreviations: β-APP, β-amyloid precursor protein; CSF-1 or M-CSF, macrophage colony-stimulating factor; TGF, transforming growth factor; TNF, tumor necrosis factor; R, receptor; ACE, angiotensin-converting enzyme; TAPI, tumor necrosis factor α protease inhibitor; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; WT, wild type; TM, transmembrane domain.

[2] P. Deng and P. K. Pattengale, manuscript in preparation.

10.1021/bi9817313 CCC: $15.00
Published on Web 11/20/1998

cleavage and their mode of activation. The proteolytic enzyme catalyzing the ectodomain cleavage of TNF-α (called the TNF-α-converting enzyme or TACE) (*23*, *24*) has recently been cloned; however, it is not known whether this enzyme is also responsible for the ectodomain cleavage of other cell surface proteins such as CSF-1^{256}, TGF-α, and *β*-APP. Ectodomain cleavage occurs at or near the cell surface and is typically within 15 amino acids from the transmembrane domain. The peptide bonds that are cleaved by the ectodomain cleavage system have been identified in a few cases (*2*, *3*, *25*−*27*). Although the cleavage sites and the amino acid sequences around the cleavage sites are quite dissimilar, the cleavage usually occurs at a single peptide bond for the respective proteins. For example, TGF-α is cleaved between residues 50 (Ala) and 51 (Val), and *β*-APP is cleaved between residues 16 (Lys) and 17 (Leu) of *β*-amyloid sequence (*2*, *28*). These observations raise the question: how is the peptide bond selected for cleavage? We have used CSF-1^{256} as a model system for the ectodomain cleavage of cell surface transmembrane proteins. We have previously shown that native conformation and dimer formation of the growth factor domain are not required for the cleavage of CSF-1^{256} (*29*) and that multiple factors specified by the juxtamembrane region determine the cleavage efficiency (*30*). In an effort to better characterize the ectodomain cleavage of CSF-1 and to further identify the mechanisms by which the cleavage site of CSF-1^{256} is selected, we have therefore studied the effect of various deletion, insertion, and substitution mutations at or near the cleavage site on both the number and size of cleaved CSF-1^{256} products as measured by SDS−PAGE. We have shown that cleavage site selection is dependent on the amino acid sequence of the juxtamembrane region, the distance of the site from the transmembrane domain, and steric accessibility of the protease.

EXPERIMENTAL PROCEDURES

(I) 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 units/mL), and streptomycin sulfate (100 μg/mL).

(II) Site-Directed Mutagenesis and Stable Transfection. The human WT CSF-1^{256} cDNA in pBluescriptIISK(PD)-CSF-1^{256} (*30*, *31*) was used as the template for the mutagenesis of CSF-1. The PCR mutagenesis technique of Ho et al. (*32*) was used for generation of one insertion mutation (CSF-1^{256}-162FAE163) and two truncation mutations (CSF-1^{256}-Stop150 and CSF-1^{256}-Stop159) (Figure 1B). The constructions of other mutations were described previously (*30*). All sequences produced by PCR were confirmed by DNA sequencing with Sequenase (U.S. Biochemical Corp.). The *Xho*I fragments containing the mutant cDNA forms in the BluescriptIISK(PD) vectors (*30*) were subcloned into the *Xho*I site in an expression retroviral vector, PSM (*33*).

Expression vectors containing genes for CSF-1 mutants were transfected into NIH 3T3 cells by the calcium phosphate precipitation technique. Transfectants were subcloned after selection in 600 μg/mL G418. Immunoprecipitation (see below) was used to identify clones expressing mutant CSF-1 forms.

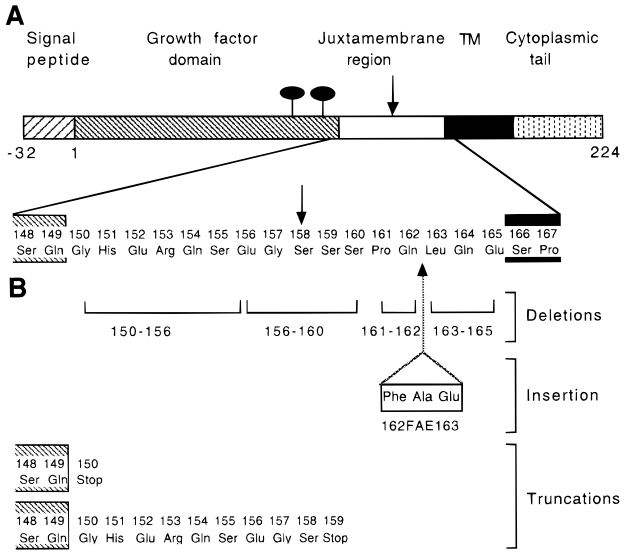

FIGURE 1: Schematic representation of the human CSF-1^{256} proteins. (A) Domain structure and juxtamembrane sequence of human CSF-1^{256}. Amino acids are numbered from the amino-terminus of the soluble growth factor. Canonical sites for addition of N-linked glycosylation are indicated by solid ovals.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

(III) Metabolic Labeling and Immunoprecipitation. Cells expressing WT or mutant CSF-1 forms were metabolically labeled with [^{35}S]methionine for 20 min or 1 h and chased with complete medium containing 20× methionine for 1 h, allowing the expression of labeled CSF-1^{256} on the cell surface. Then the ^{35}S label was chased in the same medium for 3 h in the presence or absence of 0.5 μM phorbol 12-myristate 13-acetate (PMA) (Sigma) as indicated. The medium was collected for immunoprecipitation by the YYG-106 rat monoclonal antibody (*34*). Immunoprecipitation was carried out with protein A−Sepharose (Pharmacia, Piscataway, NJ) precoated with a goat anti-rat IgG (Cappel) as the immunoadsorbant. The control for nonspecific precipitation was performed with an isotype-matched rat myeloma protein. The amounts of washed immune precipitates were adjusted to produce comparable signal intensities, and the samples were subjected to SDS−12.5% polyacrylamide gel electrophoresis (PAGE) under reducing or nonreducing conditions. The labeled proteins were detected by fluorography and quantitated with Betascope 603 (Betagen). The apparent molecular weights of the labeled proteins were determined by comparison with the mobilities of protein molecular weight standards. For metabolic labeling under tunicamycin treatment, cells were preincubated for 4 h in medium with tunicamycin (2 μg/mL), labeled, and chased in the continued presence of the drug.

(IV) N-Glycanase Digestion. N-Glycanase digestion was performed as previously described (*35*). Briefly, collected medium containing radiolabeled soluble CSF-1 was immunoprecipitated and washed precipitates were incubated for 18 h at 37 °C with 0.3 unit of N-glycanase (Genzyme). The incubation was carried out in a 20 μL volume of digestion buffer containing 100 mM sodium phosphate (pH 8.6), 10 mM dithiothreitol, 10 mM EDTA, 0.1% SDS, and 1% Triton X-100. Washed immunoprecipitates were heated in digestion buffer for 30 min at 60 °C before the addition of the enzyme.

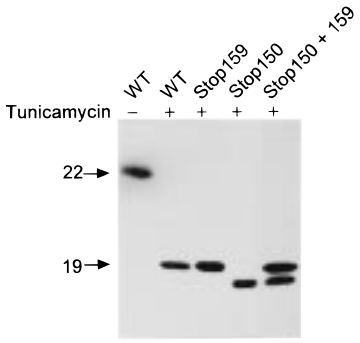

FIGURE 2: Resolution of soluble CSF-1 forms by SDS−PAGE. NIH 3T3 cells stably transfected with either WT CSF-1^{256} or mutant CSF-1^{256} forms were metabolically radiolabeled for 1 h and chased for 1 h for maturation in the presence (+) or absence (−) of tunicamycin. Then the cells were chased for 3 h in medium with 0.5 μM PMA for WT CSF-1^{256} or in medium without PMA for both CSF-1^{256}-Stop150 and CSF-1^{256}-Stop159. The culture medium was immunoprecipitated and separated by SDS−PAGE with disulfide reduction. The apparent molecular masses of CSF-1 molecules are noted in kilodaltons at the left margin.

RESULTS

(I) Strategy for Analysis of the CSF-1 Ectodomain Cleavage Site. The CSF-1^{256} precursor is composed 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 (Figure 1A). WT CSF-1^{256} is synthesized as a cell surface homodimeric glycoprotein with a 34-kDa subunit. It is stably expressed on the cell surface and is slowly cleaved, releasing soluble homodimeric CSF-1 with a 22-kDa subunit (Figure 2) (*20*). The cleavage can be accelerated by PMA (*8*). Amino acid sequence determinations of cleaved CSF-1 from conditioned media of SV40-infected CV-1 monkey cells transfected with CSF-1^{256} cDNA have previously demonstrated that the cleavage site is at or near residue 158 (Figure 1A) (*36*). Deletion of region 156−160 containing the cleavage site still allows for the cleavage (*30*), which indicates that an altenative cleavage site can be utilized. Therefore, we hypothesized that certain mutations in the juxtamembrane region might cause changes in cleavage site selection and took the approach of detecting the changes by comparing both the number and size of cleaved CSF-1 products derived from mutant CSF-1 forms with those derived from WT CSF-1. Being N-linked glycosylated, soluble CSF-1 appeared as a broad diffuse band of 22 kDa (Figure 2), and thus, it was not suitable for resolving the small differences among different CSF-1 forms. When glycosylation of the protein was prevented by treating CSF-1-expressing cells with tunicamycin, an inhibitor of N-linked glycosylation (*37*), the cleaved CSF-1 was resolved as a much sharper band of 19 kDa (Figure 2). Furthermore, variant protein forms with a few amino acid differences are more readily resolved when molecular masses of the proteins are smaller. Therefore, the changes in cleavage site selection were assayed by using tunicamycin treatment of CSF-1-expressing cells.

To determine how many amino acid differences of soluble CSF-1 can be resolved by our gel system, we used two truncated CSF-1 mutants, CSF-1^{256}-Stop150 and -Stop159 (Figure 1B), as reference sizes. CSF-1^{256}-Stop150 and -Stop159 encode CSF-1 polypeptides of 181 and 190 residues, respectively, which include the 32 amino acid signal peptide and residues 1−149 and residues 1−158 of the CSF-1 amino-terminal domain (Figure 1B). In the presence of tunicamycin, soluble CSF-1 derived from cultures producing CSF-1^{256}-Stop159 was virtually identical in size to that generated by ectodomain cleavage of WT CSF-1 (Figure 2). This result is consistent with the finding that CSF-1^{256} is cleaved at or near residue 158 (*36*). Soluble CSF-1 derived from cultures producing CSF-1^{256}-Stop150 migrated as a smaller molecule. The difference of nine amino acids between these two CSF-1 forms was clearly resolved by our gel system (Figure 2). On the basis of the separation of these two bands, which differed by nine amino acids, it appears that the limit of the resolution of this gel system is about two amino acids (Figure 2).

(II) The CSF-1^{256} Cleavage Site Is Specified by Distance between the Cleavage Site and the Plasma Membrane. To define the structural requirements for ectodomain cleavage of CSF-1, we previously introduced small deletion and substitution mutations around the cleavage site and stably transfected the altered cDNAs into NIH 3T3 cells. We showed that the structural requirements for efficient ectodomain cleavage involve an essential protease binding site, a cleavage site, and steric accessibility of the proteolytic enzyme, all of which are determined by the extracellular juxtamembrane region (residues 150−165) of CSF-1^{256} (*30*). In this study, we utilized the mutants that still yielded soluble CSF-1 to determine how the cleavage site is selected by the proteolytic system. NIH 3T3 cells expressing WT CSF-1^{256} or various mutant CSF-1 cDNAs were metabolically labeled in the presence of tunicamycin for 1 h followed by exposure to PMA for another 3 h to induce processing. The media were collected and immunoprecipitated, and the washed products were analyzed by SDS−PAGE under reducing conditions. Deletion of region 161−162 (Δ161−162) or region 163−165 (Δ163−165), C-terminal to the cleavage site (residues 157−159), was found previously to dramatically decrease the cleavage efficiency (Table 1) (*30*). In this study, these two deletions yielded one smaller cleavage product (Figure 3A, Table 1). Compared with the reference sizes of CSF-1^{256}-Stop150 and -Stop159, the difference in electrophoretic migration pattern indicated that the products of CSF-1^{256}-Δ161−162 and -Δ163−165 are about two amino acids smaller than that of WT CSF-1^{256}. This difference in migration can be explained by cleavage occurring not at the original site (residues 157−159) but, rather, at a site N-terminal to the original site. These results strongly suggest that cleavage occurs at a defined distance from the transmembrane domain. Consistent with this finding, deletion of region 150−156 (Δ150−156), N-terminal to the cleavage site, yielded one smaller cleavage product (Figure 3A, Table 1). Although these electrophoretic approaches did not allow precise determination of the site of the cleaved peptide bond, the changes in cleavage site selection were clearly demonstrated.

(III) The CSF-1^{256} Cleavage Site Is Specified by the Amino Acid Sequence of the Juxtamembrane Region. To further test the distance dependence of the cleavage, an insertion mutant (CSF-1^{256}-162FAE163) adding three additional residues (Phe-Ala-Glu) between the normal cleavage site and the transmembrane domain (i.e., between Gln^{162} and Leu^{163}) in WT CSF-1^{256} was constructed and stably expressed in NIH 3T3 cells (Figure 1B). It was found that this insertion did not

Table 1: Ectodomain Cleavage Efficiency and Cleavage Site Selection of CSF-1^{256} Forms with Mutations in the Juxtamembrane Region

	cleavage efficiency[a]	number of products[b]	size[b] (kDa)
WT	++++	1	19
	Deletions		
Δ150−156[c]	+	1	<19
Δ156−160	++	1	19
Δ161−162	±	1	<19
Δ163−165	±	1	<19
	Insertion		
162FAE163	++++	3	19, >19, ≫19
	Substitutions		
Ala158−160	+++	1	19
Asp158−160	+	1	<19
Leu158−160	++	2	19, >19
Lys158−160	++	1	<19
Pro161Ala	+++	1	19
Pro161Gly	++	1	19
Pro161Ser	++	1	19
Gln162Glu	++++	1	19
Gln162Lys	++++	1	19
Gln162Pro	++++	1	19
Leu163Pro	++++	1	19
Leu163Ile,Gln164Pro	+	1	19
Gln164Glu	++++	1	19
Gln164Lys	++++	1	19
Glu165Gln	++++	1	19

[a] NIH 3T3 cells stably transfected with either WT CSF-1^{256} or mutant CSF-1^{256} 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 immunoprecipitated and subjected to SDS−PAGE under nonreducing conditions followed by quantitative fluorography. The cleavage efficiency of CSF-1^{256} is represented by symbols: the portion cleaved was <2% (−), 2−10% (±), 10−30% (+), 30−70% (++), 70−90% (+++), or >90% (++++). [b] Cells were metabolically radiolabeled for 1 h and chased for 1 h for maturation in the presence of tunicamycin. Then the cells were chased in medium with 0.5 μM PMA for 3 h. The culture medium was immunoprecipitated and separated by SDS−PAGE with disulfide reduction. The number of the cleaved CSF-1 products is counted, and the sizes of the products are noted: 19 (equal to the size of WT), <19 (smaller than the size of WT), >19 (larger than the size of WT), or ≫19 (much larger than the size of WT). [c] Without tunicamycin treatment, CSF-1^{256}-Δ150−156 yielded two smaller products of similar amount as shown in Figure 4.

decrease the cleavage efficiency (Table 1). We expected that this insertion would yield only one product with a retarded migration relative to that of WT CSF-1^{256} according to the model of distance specificity. Contrary to our expectation, this mutant yielded three products (Figure 3B). The size of the major product was equal to that of WT, suggesting the cleavage of this product occurred at the original cleavage site. Detection of the other two larger products suggested that the cleavage sites of this mutant are dependent on both the amino acid sequence and its distance from the membrane. Similarly, a deletion encompassing the cleavage site, CSF-1^{256}-Δ156−160, yielded one product of the same size as that of WT (Figure 3B), which also suggested that distance constraint is not the only feature that specifies the cleavage site, and some other features, such as amino acid sequence specificity, are also involved in determining the cleavage site.

We also analyzed the effects of the substitutions in the juxtamembrane region on the cleavage site selection. Three of the four substitutions at the cleavage site caused cleavage site changes. CSF-1^{256}-Leu158−160 yielded two products:

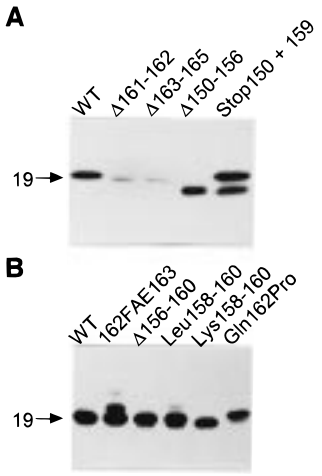

FIGURE 3: Effect of mutations in the juxtamembrane region on the cleavage site selection of CSF-1^{256}. Cells were metabolically radiolabeled for 1 h and chased for 1 h for maturation in the presence of tunicamycin. Then the cells were chased in medium with 0.5 μM PMA for 3 h. The culture medium was collected and immunoprecipitated. The amounts of precipitated products were adjusted to produce comparable signal intensities, and the samples are separated by SDS−PAGE with disulfide reduction. The apparent molecular masses of CSF-1 molecules are indicated in kilodaltons at the left margin.

the major one was equal to that of WT CSF-1^{256}, and the other minor one was larger (Figure 3B, Table 1). In addition, CSF-1^{256}-Lys158−160 (Figure 3B, Table 1) and CSF-1^{256}-Asp158−160 (Table 1) yielded one smaller product than that of WT CSF-1^{256}. However, CSF-1^{256}-Ala158−160 yielded one product of the same size as that of WT CSF-1^{256} (Table 1). In contrast, all the 11 substitution mutants in the PQLQE region including CSF-1^{256}-Gln162Pro (Figure 3B, Table 1), and the other 10 mutants (Table 1) yielded molecules of the same size as that of WT CSF-1^{256}, although the cleavage efficiency of each mutant was different (Table 1) (*30*). These results demonstrate that the amino acid sequence of the juxtamembrane region and the distance from the transmembrane domain are both involved in determining the cleavage site. In addition, the data in Table 1 indicate that the cleavage efficiency is not simply correlated with the cleavage site that is used.

(IV) The CSF-1^{256} Cleavage Site Is Specified by the Accessibility of the Protease(s). CSF-1^{256} is a heavily glycosylated protein that contains only N-linked oligosaccharides with terminal sialic acids (*35*). The canonical sugar attachment sites (Asn^{122} and Asn^{140}) are located near the cleavage site (Figure 1A). We have previously shown that N-glycosylation of certain CSF-1 mutants with shortened length of the juxtamembrane region such as CSF-1^{256}-Δ150−156 sterically interfere with protease accessibility and therefore reduce the cleavage efficiency (*30*). In this study, we asked whether the accessibility of the protease also determines the cleavage site selection. We compared the cleavage site changes in mutant CSF-1^{256} forms under tunicamycin treatment with those changes without tunicamycin treatment. The CSF-1 forms produced without tunicamycin treatment were subsequently deglycosylated with N-glycanase (*38*) to facilitate the comparison of the number and size of mutant CSF-1 polypeptides released from cells without tunicamycin treatment with both the number and size of the mutant CSF-1 polypeptides released from cells with

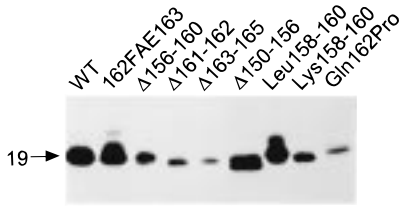

FIGURE 4: Effect of steric hindrance of protease accessibility on the cleavage site selection of CSF-1^{256}. NIH 3T3 cells were metabolically radiolabeled for 1 h and chased for 1 h for maturation in the absence of tunicamycin. Then the cells were chased in medium containing 0.5 μM PMA for 3 h. The chasing medium was precipitated with antibody to CSF-1, and the immune complexes were incubated in the presence of N-glycanase. The amounts of precipitated products were adjusted to produce comparable signal intensities, and the samples were resolved by SDS−PAGE with disulfide reduction followed by autoradiography of the dried gel.

tunicamycin treatment. WT CSF-1^{256} yielded soluble CSF-1 of the same size in the presence or absence of tunicamycin treatment (Figures 3 and 4, Table 1). Mutations including CSF-1^{256}-Δ161−162, CSF-1^{256}-Δ163−165, CSF-1^{256}-162FAE163, and CSF-1^{256}-Leu158−160 led to similar changes in both the size and number of cleaved CSF-1 products (Figures 3 and 4). In marked contrast, CSF-1^{256}-Δ150−156 yielded two smaller products in similar amounts without tunicamycin treatment (Figure 4) while producing only one smaller product with tunicamycin treatment (Figure 3A). These results suggest that N-glycosylation of this mutant has an effect on cleavage site selection by sterically interfering with the protease accessibility.

DISCUSSION

Many cell surface transmembrane proteins involved in various aspects of cell communication and regulation undergo proteolytic cleavage to release their ectodomains into the extracellular compartment. The structural requirements for the ectodomain cleavage have been identified in a few cases. The available information about cleavage of the ectodomain proteins suggests that the same rules do not apply to all of them. More and more lines of evidence suggest that the extracelluar juxtamembrane region is critical for the cleavage efficiency(*25, 30, 39*−*43*). It was demonstrated that the presence or absence of the cytoplasmic domains of IL-6R (*39*), p60 TNFR (*40*), β-APP (*44*), kit ligand (*45*), CSF-1^{256} (*30*), and ACE (*46*) had no effect on the ectodomain cleavage of these proteins, whereas the cytoplasmic tail of TGF-α (*47*)and the p80 TNFR (*48*) is required for the cleavage of these two proteins. More recently, the distal ectodomain of ACE has been suggested to regulate its cleavage (*46*). In contrast, we showed that CSF-1 molecules with cysteine mutations in the distal extracellular growth factor domain still underwent efficient ectodomain cleavage, despite undergoing conformational changes resulting in marked alterations in their monoclonal antibody recognition, dimer formation, transport, and biological activity (*29*). Regardless of structural requirements for cleavage efficiency, these proteins are usually cleaved at preferred cleavage sites in the juxtamembrane region, which show no obvious amino acid sequence similarities (*2, 3, 25*−*27*). Little, however, is known of the mechanism by which the cleavage sites are selected.

It has been suggested that the selection of the cleavage site of certain proteins such as β-APP (*49*) and IL-6R (*39*) is determined by the distance from the transmembrane domain. In marked contrast, Ehlers et al. (*26*) reported that ectodomain cleavage of membrane-bound ACE is not constrained by a defined distance from the membrane or by a specific cleavage site motif, and they proposed that the cleavage of ACE is primarily determined by the distance to the proximal extracellular domain. We have used CSF-1^{256} as a model system for studying the ectodomain cleavage of cell surface transmembrane proteins. We have previously shown that native conformation and dimer formation of the CSF-1^{256} distal growth factor domain is not required for the cleavage (*29*) and that the cleavage efficiency is determined by multiple factors including an essential protease binding site, a cleavage site, and steric accessibility of the proteolytic enzyme (*30*). In the present report, we show that the amino acid sequences of the juxtamembrane region, distance from the cleavage site to plasma membrane, and protease accessibility jointly determine the selection of the cleavage site where the release of the CSF-1^{256} extracellular growth factor domain occurs. Furthermore, our previous data and the data here demonstrate that the structural determinants for the cleavage efficiency and those for the cleavage site selection are not the same.

Deletion of two or three amino acids between the cleavage site and the plasma membrane (CSF-1^{256}-Δ161−162, CSF-1^{256}-Δ163−165) brings the original cleavage site two or three amino acids closer to the plasma membrane. These two mutants undergo cleavage at a new site, about two amino acids N-terminal to the original cleavage site, suggesting distance specificity determines the cleavage site selection by keeping the cleavage site at the same distance from the transmembrane domain in these two deletion mutants. Deletion of region 150−156 (seven amino acids) between growth factor domain and the cleavage site keeps the original cleavage site at the same distance from the plasma membrane, while bringing the growth factor domain seven amino acids closer to the original cleavage site. This mutant yielded a single product that was about seven amino acids smaller than that of WT CSF-1^{256}, which is consistent with the distance specificity. This finding agrees with the cleavage site selection of β-APP and IL-6 receptor (*39*, *49*).

Insertion of three amino acids (Phe-Ala-Glu) (CSF-1^{256}-162FAE163) between the cleavage site and the plasma membrane places the original cleavage site three amino acids more distant from the plasma membrane. If the cleavage site selection were specified by the distance dependence, this insertion would yield one product that is larger than that of WT CSF-1^{256}. However, the proteolytic cleavage system not only cleaves CSF-1^{256}-162FAE163 at the original site suggested by the size of the cleaved CSF-1, but also cleaves CSF-1^{256} at two new sites that are C-terminal to the original cleavage site. The cleavage at the original site indicates that in this case the selection of the cleavage site was determined by the amino acid sequence of the juxtamembrane region rather than by the distance from the transmembrane domain. The cleavage at the other two sites suggests that both the amino acid sequences of the juxtamembrane region and the distance from the transmembrane domain are involved in cleavage site selection. It should be noted that phenylalanine has a bulky side chain and glutamic acid is negatively

charged, and these characteristics of the insertion mutant might also contribute to the changes in the cleavage site selection of this mutant. Furthermore, substitutions of Ser158−160 at the cleavage site with lysines (positively charged), aspartic acids (negatively charged), or leucines (bulkier side chain) also caused changes or shifts of the cleavage site, while substitution of Ser158−160 with alanines did not lead to significant changes in the cleavage site selection. Taken as a whole, these experiments provide additional evidence for the role of amino acid sequence in defining cleavage site specificity.

We also tested whether distortion of the native secondary structure of CSF-1^{256} in the juxtamembrane region interferes with the selection of the cleavage site. Proline places a higher constraint on the polypeptide backbone than any other amino acid and disrupts the secondary structure features of proteins (*50*). Therefore, it might be expected that substitutions of residue Pro^{161} with alanine, glycine, or serine, substitutions of residue Gln^{162} or Leu^{163} with proline, and substitution of $Leu^{163}Gln^{164}$ with isoleucine-proline would have profound effects on bending the distal cleavage site toward or away from the cell surface, causing significant changes in cleavage site selection according to distance specificity. However, these substitutions did not lead to significant changes in cleavage site selection. These findings suggest that maintenance of the native secondary structure of CSF-1^{256} in the juxtamembrane region is not required for cleavage site selection.

We have previously shown that steric hindrance of protease accessibility decreased the cleavage efficiency of certain CSF-1 mutants (*30*). In this study, differential cleavage site selection of CSF-1^{256}-Δ151−156 was identified in cells in the presence or absence of tunicamycin treatment, indicating that N-glycosylation of this mutant is involved in the determination of the cleavage sites in this case. Furthermore, N-glycosylation of WT CSF-1^{256} and other mutant CSF-1 forms did not affect the selection of the cleavage sites, suggesting that N-glycosylation affects the cleavage site selection by sterically interfering with the accessibility of the protease, rather than by a specific interaction between the carbohydrate and the protease. Therefore, a mechanism of steric hindrance, which likely involves interference with protease accessibility, was able to cause not only the decrease in the cleavage efficiency but also the change in the cleavage site selection in certain CSF-1 mutants. This mechanism of steric hindrance might provide an alternative explanation for the cleavage site selection of certain cell surface proteins such as ACE, in which a minimum distance is required between the cleavage site and the first, proximal extracellular domain (*26*).

Recently, Sadhukhan et al. (*46*) demonstrated that, in a chimeric protein of ACE/CD4, the distal extracellular domain in cleavable ACE can induce an ectodomain cleavage within the juxtamembrane domain of uncleavable CD4. The authors speculated that the distal extracellular domain of ACE contains a specific structure to activate the ectodomain cleavage. By inference from our findings, an alternative explanation would be that the overall structure of the distal extracellular domain of CD4 prevents the ectodomain cleavage in the juxtamembrane region by steric hindrance of protease accessibility. Switching to the ACE extracellular domain allows for the accessibility of the protease to the juxtamembrane region of CD4, thereby permitting efficient cleavage.

The results in our previous study strongly suggest that the extracellular growth factor domain of CSF-1^{256} is cleaved and released into the extracellular compartment by a membrane-associated proteolytic enzyme (or enzymes) (*30*). This study showed that ectodomain cleavage of CSF-1^{256} occurs at a preferred distance from the plasma membrane, suggesting that the protease responsible for the cleavage is physically constrained by its association with the plasma membrane, therefore providing an additional line of evidence that the protease(s) involved is membrane-bound.

ACKNOWLEDGMENT

We are grateful to Dr. Carl W. Rettenmier, in whose laboratory these studies were initiated. We thank Drs. Julius Peters and Kirston Koths for helpful discussions and critical reading of the manuscript.

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BI9817313