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# Cleavage of a Hydrophilic C-Terminal Domain Increases Growth-Inhibitory Activity of Oncostatin M

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**Oncostatin M is a polypeptide cytokine, produced by normal and malignant hematopoietic cells, that has several in vitro activities, including the ability to inhibit growth of cultured carcinoma cells. Here we present a structural and functional comparison of two oncostatin M-related proteins ($M_r$ 36,000 and 32,000) secreted by COS cells transfected with oncostatin M cDNA. The smaller of these forms lacked a hydrophilic C-terminal domain comprising predominantly basic amino acids. This domain was also absent from native oncostatin M produced by U937 cells. The 32,000-$M_r$ form of oncostatin M was not produced by cells transfected with plasmids (G195 and G196) in which a potential trypsinlike cleavage site within the hydrophilic C-terminal domain was altered by site-directed mutagenesis. A 32,000-$M_r$ fragment was produced by trypsin treatment of the 36,000-$M_r$ form of oncostatin M. These observations suggest that the 32,000-$M_r$ form of oncostatin M was derived from the 227-amino-acid propeptide by proteolytic cleavage at or near the paired basic residues at positions 195 and 196. Pro-oncostatin M was equally active in radioreceptor assays as the processed form but was 5- to 60-fold less active in growth inhibition assays. Likewise, nonprocessed mutant protein encoded by plasmid G196 was equally active in the radioreceptor assays as the processed form but was five- to ninefold less active in growth inhibition assays. Thus, the highly charged C-terminal domain of pro-oncostatin M is not required for receptor binding or growth-inhibitory activity but may alter the functional properties of the molecule. Propeptide processing of oncostatin M may be important for regulating in vivo activities of this cytokine.**

During an inflammatory response, cells of the immune system release numerous cytokines and lymphokines (8, 22). Although many of these factors are structurally distinct, they sometimes have similar properties in vitro, such as the ability to directly regulate cellular growth (4, 7, 25, 29). Some cytokines may also perform related functions in vivo (8). In many cases, the expression of a cytokine is regulated by other cytokines, leading to the concept of a cytokine network (14). It is not surprising, therefore, that cytokine regulation is a complex process involving transcriptional (30), posttranscriptional (26), and posttranslational (1, 12) controls.

Oncostatin M is a novel cytokine produced by lymphoid cells (3, 21, 23), which was originally identified for its ability to inhibit growth of the A375 melanoma cell line (3, 33). Subsequent cDNA-cloning studies showed that oncostatin M is a low-molecular-weight secreted glycoprotein with no significant sequence homology with other known proteins (21). Like most other polypeptide cytokines, oncostatin M appears to initiate its biological activities by binding to specific cell-surface receptors present on responsive cells (18; D. Horn, W. C. Fitzpatrick, P. T. Gompper, V. Ochs, M. Bolton-Hansen, Z. Zarling, N. Malik, G. T. Todaro, and P. S. Linsley, Growth Factors, in press). Although the importance of oncostatin M in vivo has yet to be established, it has several in vitro activities, including growth regulation of many tumor and normal cell lines (Horn et al., in press) and regulation of plasminogen activator levels and growth of cultured endothelial cells (T. J. Brown, J. M. Rowe, M. Shoyab, and P. Gladstone, *in* R. Roberts and J. Sambrook, ed., *Molecular Biology of the Cardiovascular System*, in press).

During our initial studies on the expression of recombinant oncostatin M in mammalian cells, we noted that transfection of oncostatin M cDNA into COS cells resulted in production of two proteins ($M_r$, 32,000 and 36,000) that were immunologically related to oncostatin M (21). We have now investigated these different forms of oncostatin M and here report on their structural and functional properties.

## MATERIALS AND METHODS

**Cell culture.** A375, H2981, and COS cells were cultured as described previously (18), in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum.

**Preparation of antisera.** Peptides corresponding to amino acids 6 through 19 and 206 through 218 of oncostatin M (numbered from the N-terminal alanine of natural and recombinant oncostatin M; see Fig. 2) were synthesized by solid-phase techniques. Peptides were conjugated to bovine immunoglobulin (peptide 6-19) or keyhole limpet hemocyanin (peptide 206-218), and rabbits were immunized as described previously (10, 19).

**Mutant construction.** The oncostatin M cDNA expression plasmid pSPOM has been described previously (21), as has an expression plasmid (18; herein referred to as pβOM) in which the oncostatin M signal sequence was replaced with a signal sequence from simian transforming growth factor β1 (TGFβ1).

Deletion mutant Δ190 was constructed by limited exonuclease digestion (13) from the 3′ end of the oncostatin M-coding region. Briefly, the oncostatin M cDNA was subcloned into the plasmid pSP64 (Promega Biotec Co.), linearized near the 3′ end of the cDNA, and subjected to limited digestion with exonuclease III. The 3′ ends of the digested cDNAs were next blunted with the Klenow fragment of DNA polymerase I. Truncated cDNAs were then excised from pSP64 at an engineered *Hin*dIII site 37 bases 5′ to the translation start site. Finally, cDNAs were cloned into

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*Hin*dIII-*Xho*I-cleaved πH3MPy (27) by using synthetic linker oligonucleotides TAGGTGAATGATCAC and TCG AGTGATCATTCACCTA, which encode stop codons in each reading frame and have an overhanging end complementary to the *Xho*I restriction site. A clone encoding oncostatin M terminating at position 190 (Δ190) was identified by DNA sequence analysis (23).

Mutant clones G195 and G196 were constructed by oligonucleotide-directed mutagenesis with a commercial kit (Amersham Corp.). Mismatched oligonucleotides directing the conversion of arginine residues at position 195 or 196 into glycines were synthesized and used as specified by the supplier to construct mutant clones. Sequences of the mutated regions of clones G195 and G196 were confirmed by DNA sequence analysis.

Mutant clones Δ182 and Δ195 were constructed by a modification of the oligonucleotide mutagenesis procedure. After repolymerization and ligation of gapped M13 phage DNA, oncostatin M cDNA was amplified by using Tac polymerase and the polymerase chain reaction (The Perkin Elmer Corp.) with M13 universal forward and reverse primers. The amplified cDNA was then subcloned into the *Hin*dIII-*Xho*I-cleaved πH3MPy, and mutant clones were identified by restriction analysis and/or DNA sequence analysis. The sequences of the mutated regions were then confirmed by sequence analysis. Further sequence analysis of Δ195 revealed that the alanine at position 2 of oncostatin M had been substituted by a valine as a result of a secondary mutation introduced during mutagenesis.

**Transient expression of oncostatin M.** COS cells (11) were transfected with pSPOM, pβOM, or mutant plasmids as previously described (21). At 24 h after transfection, serum-free medium was added, and cells were incubated at 37°C for an additional 48 h. Media were collected and, where indicated, assayed immediately. Alternatively, media were acidified by the addition of acetic acid to 1 N and concentrated for purification of oncostatin M. Since there was no detectable difference in relative levels or activities of different forms of oncostatin M produced by pβOM- or pSPOM-transfected cells, these were used interchangeably.

**Purification of oncostatin M.** The 32,000-$M_r$ form of oncostatin M was purified essentially as described previously (18, 21) from acidified and concentrated serum-free medium from COS cells transfected with pSPOM. Peak fractions of growth-inhibitory activity from size-fractionated culture medium were collected and subjected to final purification by reversed-phase chromatography. As reported previously (18, 21), oncostatin M preparations prepared in this fashion contained predominantly the 32,000-$M_r$ form of oncostatin M.

Oncostatin M used for radiolabeling with $^{125}I$ or for standard curves for quantitative immunoblotting was expressed in CHO cells (N. Malik, J. Kallestad, and P. S. Linsley, unpublished observations). Serum-free medium (a gift of N. Malik) was collected, and oncostatin M was purified as described above. Oncostatin M purified from this source does not show immunoreactivity with anti-206-218 serum (see below) and has properties equivalent to the 32,000-$M_r$ form of oncostatin M produced by COS cells.

The 36,000-$M_r$ form of oncostatin M from pSPOM-transfected cells was partially purified in a three-step procedure. Serum-free culture medium was acidified and size fractionated on a TSK 3000SW column run in 40% acetonitrile–0.1% trifluoroacetic acid. Fractions containing predominantly the 36,000-$M_r$ form of oncostatin M were identified by immunoblotting analysis with anti-6-19 serum and were pooled, concentrated, and rerun over the same column. Fractions containing immunochemically pure 36,000-$M_r$ form (i.e., having no detectable 32,000-$M_r$ form) were pooled and used for subsequent experiments. Unprocessed oncostatin M from G196-transfected cells was purified by two cycles of a two-step procedure consisting of an initial reversed-phase chromatography step followed by size fractionation.

**GIA.** Growth-inhibitory activity was measured by a dye-binding growth inhibition assay (GIA) (18, 29). A375 melanoma cells ($3 \times 10^3$ to $4 \times 10^3$) were seeded in a volume of 0.1 ml of DMEM containing 10% fetal bovine serum in 96-well microdilution plates. Various concentrations of oncostatin M were added in a volume of 0.1 ml, and incubation at 37°C was continued for 72 h. The culture medium was removed, and relative cell proliferation was measured as described previously (18). Cellular proliferation in the presence of oncostatin M was compared with proliferation in untreated samples and is expressed as a percentage of inhibition of maximal (untreated) growth. Samples were assayed in duplicate or triplicate and generally varied by less than 10% from each other. GIA units are defined to be the amount of protein needed to inhibit by 50% the growth of A375 cells in a standard assay.

**RRA.** H2981 cells were seeded at a density of $1 \times 10^5$ to $3 \times 10^5$ cells per $cm^2$ in 48-well plastic dishes 16 to 24 h before the start of the experiment. Monolayers were incubated with $^{125}I$-labeled oncostatin M (~20 ng/ml, 0.7 nM) in 0.1 ml of binding buffer (18) containing increasing amounts of unlabeled oncostatin M. Binding reactions were carried out for 2 to 4 h at 23°C. Nonspecific binding was measured in the presence of a 50- to 100-fold excess of unlabeled oncostatin M and amounted to 5 to 30% of the total. Variation between replicate determinations was generally less than 10%. One radioreceptor assay (RRA) unit is defined as the amount of oncostatin M required for 50% inhibition of binding of $^{125}I$-labeled oncostatin M in a standard assay.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the Laemmli system (17). Linear acrylamide gradient gels with stacking gels of 5% acrylamide were used. Samples were run under reducing conditions. For photography, gels were stained with Coomassie blue, destained, and dried. Immunoblotting analysis was performed as previously described (21). Both anti-6-19 and anti-206-218 sera were used at a final dilution of 1:50. Apparent molecular weights of different forms of oncostatin M were calculated by comparison with standards as described previously (21).

***N*-Glycanase treatment.** Serum-free conditioned medium was collected from COS cells transfected with pSPOM and dialyzed versus 1 N acetic acid. Two samples of acidified and dialyzed medium (0.4 ml each) were then taken to dryness by evaporative centrifugation. Proteins were solubilized in a volume of 10 μl of solution A (0.5% SDS, 0.1 M 2-mercaptoethanol), and samples were heated to 95°C for 3 min. Samples were cooled, and 18 μl of solution B (20 mM sodium phosphate [pH 8.6], 10 mM EDTA, 1% Nonidet P-40) was added. To one tube, 1 μl (0.25 U) of *N*-glycanase (Genzyme) was added, and both samples were incubated at 37°C for 16 h. Reactions were stopped by the addition of concentrated electrophoresis sample buffer, and samples were analyzed by SDS-PAGE and immunoblotting analysis.

**Oncostatin M quantitation.** Oncostatin M concentrations in impure preparations were measured by quantitative desitometry. For determination of concentrations in partially purified preparations, serial dilutions of samples were subjected to SDS-PAGE and immunoblotting analysis by using

alkaline phosphatase-conjugated protein A for detection. Blots were scanned by reflected light with a densitometer (Hoefer Scientific Instruments). Arbitrary amounts of oncostatin M were determined by paper weighing of appropriate peaks from the densitometer tracings. Arbitrary amounts were then converted to concentrations by comparison with peak weights obtained from known amounts of purified oncostatin M. Peak weights were linear over at least three serial twofold sample dilutions. Values reported represent the mean of concentrations determined from at least two different sample dilutions and had coefficients of variation of ≤25%.

Concentrations of oncostatin M in serum-free culture media were determined in a modification of the procedure described above. Serum-free culture media were diluted in DMEM, dithiothreitol was added to a concentration of 10 mM, and proteins were denatured by boiling. Serial dilutions of treated medium were next applied to a nitrocellulose membrane through a slot-blot apparatus (Millipore Corp.). Membranes were then subjected to immunoblotting analysis with anti-6-19 serum and $^{125}$I-labeled protein A for detection as described previously (19). Autoradiograms were scanned by densitometer by using transmitted light, and peaks were quantitated by paper weighing. Arbitrary amounts of oncostatin M were converted to concentrations by comparison to peak weights obtained with purified oncostatin M diluted in serum-free medium from mock-transfected cells. Values reported represent means (coefficients of variation of <30%) of determinations from three serial twofold sample dilutions. Dose-response curves determined with this method were linear over at least three serial twofold dilutions (corresponding to ~30 to 125 ng of oncostatin M).

Concentrations of highly purified oncostatin M preparations were determined by amino acid analysis performed by Gary Hathaway (Biotechnology Instrumentation Center, University of California, Riverside).

**Trypsin treatment of 36,000-$M_r$ oncostatin M.** The 36,000-$M_r$ form of oncostatin M was partially purified by size fractionation as described above and subjected to further purification by reversed-phase chromatography. The resulting preparation was ~80% pure as judged by SDS-PAGE. Samples containing ~150 ng of oncostatin M (estimated by RRA) were treated at 37°C with 6 ng of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington Diagnostics) in 30 μl of 50 mM Tris acetate (pH 7.9). Samples were incubated at 37°C for increasing lengths of time, reactions were terminated by the addition of concentrated electrophoresis sample buffer, and different forms of oncostatin M were identified by immunoblotting with anti-6-19 serum.

## RESULTS

**Different forms of oncostatin M produced by transfected COS cells have similar N-linked glycosylation patterns.** We previously showed (21) that medium from COS cells transfected with oncostatin M cDNA had growth-inhibitory activity on A375 melanoma cells. Medium from the transfected cells contained two proteins ($M_r$ 36,000 and 32,000) that were immunologically related, but not identical in size, to natural oncostatin M made by U937 cells (21). These same proteins were also observed when COS cells were transfected with a construct (pβOM) encoding oncostatin M with its signal sequence replaced with a signal peptide from simian TGFβ1 (21; unpublished observations). N-terminal amino acid sequencing of both the 36,000- and 32,000-$M_r$ proteins revealed the same sequence as natural oncostatin M, indicating that the difference(s) between these proteins did not result from N-terminal sequence heterogeneity.

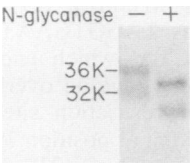


FIG. 1. Removal of N-linked oligosaccharides affects both forms of oncostatin M equally. Proteins in serum-free conditioned medium from COS cells transfected with the plasmid pSPOM were treated with the enzyme *N*-glycanase as described in Materials and Methods. Oncostatin M-related proteins were detected by immunoblotting analysis with anti-6-19 serum.

The predicted oncostatin M precursor sequence contains two potential N-linked glycosylation pattern sites (21). It was possible that differential glycosylation at these sites could account for the difference in the 36,000- and 32,000-$M_r$ forms of oncostatin M. To test this possibility, we performed the experiment shown in Fig. 1. Proteins present in serum-free conditioned medium from COS cells transfected with pSPOM were treated with the enzyme *N*-glycanase, which removes N-linked oligosaccharides, and analyzed for immunoreactivity with a site-specific antiserum to oncostatin M (anti-6-19; Fig. 2). The mobilities of both the 36,000- and 32,000-$M_r$ forms of oncostatin M were increased by this treatment, resulting in a new species of ~34,000 and 30,000 $M_r$. The increase in mobility of both fragments is consistent with the removal of one N-linked oligosaccharide moiety ($M_r$, ~2,000) from each form of oncostatin M. Since mobilities of both forms were increased in parallel, it is unlikely that differential N-linked glycosylation patterns could account for the size difference between these fragments.

**Site-specific antisera reveal different C termini for the 36,000- and 32,000-$M_r$ forms of oncostatin M.** Another pos-

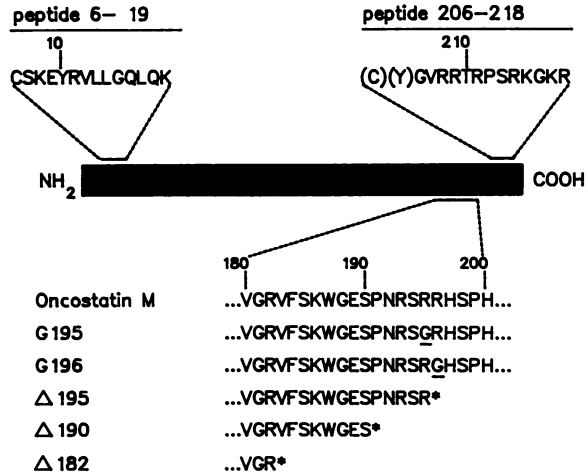


FIG. 2. C-terminal sequence of oncostatin M, showing epitopes and mutations used in this study. The sequence of oncostatin M after removal of its signal peptide (21) is represented as a bar. The positions of peptides used for generation of site-specific antisera are indicated above the bar. Parentheses indicate residues not present in oncostatin M. Below are indicated predicted protein sequences from the C terminus of oncostatin M (21) and the various mutant plasmids generated in this study. Asterisks indicate the positions of stop codons introduced into truncated forms of oncostatin M.

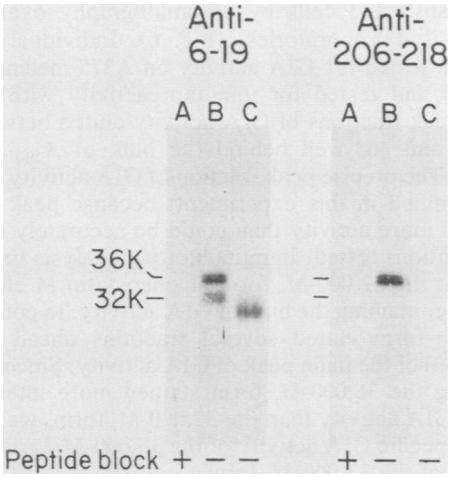


FIG. 3. Site-specific antisera detect differences in the two forms of oncostatin M from transfected cells. Identical samples (0.2 ml) of serum-free conditioned medium from COS cells transfected with the plasmid pβPOM (lanes A and B) or purified natural oncostatin M from U937 cells (~500 GIA units, lane C) were analyzed by immunoblotting analysis with the indicated antisera. Where indicated (lane A), antisera were treated with an excess of the immunizing peptide (10 μg of peptide in 40 μl of serum for 30 min at 37°C) before analysis.

sibility that could account for the size difference between the 36,000- and 32,000-$M_r$ forms of oncostatin M is C-terminal heterogeneity. Hydropathy analysis (16) revealed that the C terminus of oncostatin M (amino acids ~190 through 227) is strongly hydrophilic. Basic amino acids (R, K, or H) comprise 24 of 38 residues (63%) in this region. In addition, there are five paired basic residues that could represent potential proteolytic cleavage sites (21).

To test for C-terminal heterogeneity, we prepared antiserum to a peptide (positions 206 through 218) from the predicted C-terminus of oncostatin M (21; Fig. 2). Anti-6-19 and -206-218 sera were then used in immunoblotting experiments with serum-free conditioned medium from pSPOM transfected cells and with natural U937 oncostatin M produced by U937 cells (Fig. 3). Whereas anti-6-19 serum reacted with both the 36,000- and 32,000-$M_r$ forms from pSPOM-transfected cells (lane B) and with the 28,000-$M_r$ natural oncostatin M from U937 cells (lane C), anti-206-218 serum reacted only with the 36,000-$M_r$ form of oncostatin M (lane C). The specificity of both antisera was indicated by the ability of the cognate peptides to inhibit their reactivities (lanes A). These results indicate that differences between the 36,000- and 32,000-$M_r$ forms of oncostatin M can be accounted for, at least in part, by C-terminal heterogeneity.

**Site-specific mutations in the C-terminal hydrophilic region of oncostatin M affect accumulation of different forms of oncostatin M.** The precise C termini of the 36,000- and 32,000-$M_r$ forms of oncostatin M were unknown. Unambiguous direct C-terminal analysis of proteins is frequently a difficult procedure (31); we therefore approached this problem by introducing site-directed mutations at potential proteolytic processing sites in the C terminus of oncostatin M.

Previously published experiments had shown that the sequence of an endoproteinase Lys-C fragment of oncostatin M from U927 cells (peptide K2 [21]) terminated within a potential trypsinlike cleavage site, -R-S-R-R- (beginning at position 193). The point of sequence termination within this fragment did not correspond to a Lys-C cleavage site and

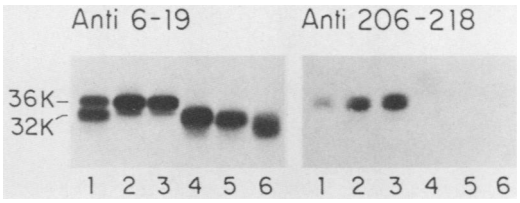


FIG. 4. Site-specific mutations reveal processing in the C-terminal hydrophilic domain of oncostatin M. COS cells were transfected with the indicated mutant plasmids, and proteins in serum-free culture medium were analyzed by immunoblotting with anti-6-19 or anti-206-218 serum. Cells were transfected with the following (lanes): 1, pSPOM; 2, G196; 3, G195; 4, Δ195; 5, Δ190; 6, 32,000-$M_r$ form purified from pSPOM-transfected cells.

was most likely not due to a limitation in the amount of fragment sequenced. One explanation for sequence termination within this fragment was that it represented the C-terminal Lys-C fragment of oncostatin M. Further support for this idea came from Chou and Fasman analysis (5) of the predicted oncostatin M sequence. This analysis predicted a beta turn structure in the region of this potential trypsinlike site, a structure that might facilitate recognition by protease(s) (data not shown).

We tested the involvement of this site in C-terminal processing by individually introducing two R-to-G mutations (arginine to glycine) at positions 195 to 196 (yielding mutant clones G195 and G196, respectively; Fig. 2). In addition, three deletion mutations were constructed in which termination codons were introduced upstream of the putative processing site (Δ182, Δ195, and Δ190). Serum-free media from COS cells transfected with several of the mutant clones were then analyzed by immunoblotting analysis (Fig. 4).

All clones encoded proteins that reacted with anti-6-19 serum. Immunoreactive proteins produced by G195 and G196 comigrated with the 36,000-$M_r$ protein from SPOM-transfected cells, whereas truncated proteins encoded by Δ195 and Δ190 migrated more closely to the 32,000-$M_r$ protein. The protein encoded by Δ182 migrated slightly ahead of the 32,000-$M_r$ form (data not shown). When anti-208-219 serum was used for analysis, only the 36,000-$M_r$ form from G195- and G196-transfected cells was detected. The proteins produced by Δ195 and Δ190 failed to react with this antiserum, as expected, since the amino acids encoding its cognate peptide were removed. Thus, introduction of point mutations at positions 195 and 196 prevented accumulation of the 32,000-$M_r$ form, suggesting that these residues comprise a major site of proteolytic processing in the 36,000-$M_r$ form of oncostatin M. Proteins from G195- and G196-transfected cells also contained a small amount of immunoreactive material that trailed to lower molecular weights (Fig. 4, lanes 2 and 3). This may have resulted from partial processing at alternative cleavage sites (i.e., cleavage at other paired basic residues in the hydrophilic C-terminal domain). This possibility was not investigated further.

**Conversion of the 36,000-$M_r$ form of oncostatin M to the 32,000-$M_r$ form by limited proteolysis.** To confirm that proteolytic processing of the 36,000-$M_r$ form of oncostatin M gives rise to the 32,000-$M_r$ form, we subjected a partially purified preparation (see below) of the 36,000-$M_r$ form to limited proteolysis (Fig. 5). Reaction products were detected with anti-6-19 serum. With increasing time of trypsinization, a gradual decrease in the amount of 36,000-$M_r$ form of oncostatin M was seen, concomitant with an increase in the amount of the 32,000-$M_r$ form. At the longest time tested

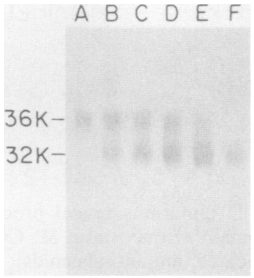


FIG. 5. Limited proteolysis of the 36,000-$M_r$ form of oncostatin M yields a 32,000-$M_r$ form. The 36,000-$M_r$ form of oncostatin M was purified and subjected to trypsin treatment as descried in Materials and Methods. Treatment at 37°C was for the following lengths of time (lanes): A, 0 min; B, 10 min; C, 20 min; D, 40 min; E, 80 min; F, 160 min.

(lane F), the amount of 32,000-$M_r$ form was decreased, and additional immunoreactive products of lower molecular weight could be observed. Since the 32,000-$M_r$ reaction product reacted with N-terminal-specific antiserum, it represented oncostatin M that had been processed at the C terminus. In other experiments, the 32,000-$M_r$ reaction product was found to have a mobility similar to that of the 32,000-$M_r$ form of oncostatin M from pSPOM-transfected cells (data not shown). These results indicate that proteolysis near the C terminus of the 36,000-$M_r$ form of oncostatin M can give rise to a 32,000-$M_r$ form.

**The 36,000-$M_r$ form of oncostatin M has reduced growth-inhibitory activity.** To determine the relative biological activities of the 36,000- and 32,000-$M_r$ forms of oncostatin M, we first fractionated serum-free conditioned medium from pβOM-transfected cells by chromatography over Bio-Gel P60 (Bio-Rad Laboratories) (Fig. 6). Individual fractions were then tested for GIA activity on A375 melanoma cells (Fig. 6A) and tested for immunoreactivity with anti-6-19 (inset). Peak fractions of GIA activity eluted between fractions 35 and 40, well behind the bulk of $A_{280}$-absorbing material. The precise peak fraction of GIA activity could not be determined in this experiment, because peak fractions contained more activity than could be accurately measured at the dilutions tested. Immunoblotting analysis (inset) indicated that the 32,000-$M_r$ form of oncostatin M eluted with fractions containing the bulk of GIA activity. In contrast, the 36,000-$M_r$ form eluted several fractions ahead (peak in fraction 33) of the main peak of GIA activity. Since fractions containing the 36,000-$M_r$ form stained more intensely but had less GIA activity than the 32,000-$M_r$ form, we conclude that the specific GIA activity of the 36,000-$M_r$ form was less than that of the 32,000-$M_r$ form.

To make quantitative comparisons of biological activities of the 32,000- and 36,000-$M_r$ forms, these forms were partially purified by size fractionation in an experiment similar to that shown in Fig. 6. For this experiment, we used a different column (TSK 3000SW), which gave results that were qualitatively similar to those with Bio-Gel P60 but offered better separation between the 36,000- and 32,000-$M_r$ forms. To minimize cross-contamination, fractions containing the 36,000-$M_r$ form of oncostatin M were pooled and subjected to a second cycle of size fractionation on the same column. Fractions containing partially purified 36,000- and 32,000-$M_r$ forms were then tested by GIA (Fig. 7A) and RRA (Fig. 7B). Oncostatin M concentrations were estimated by immunoblotting analysis and comparison with a standard amount of purified oncostatin M (Fig. 7C). The partially

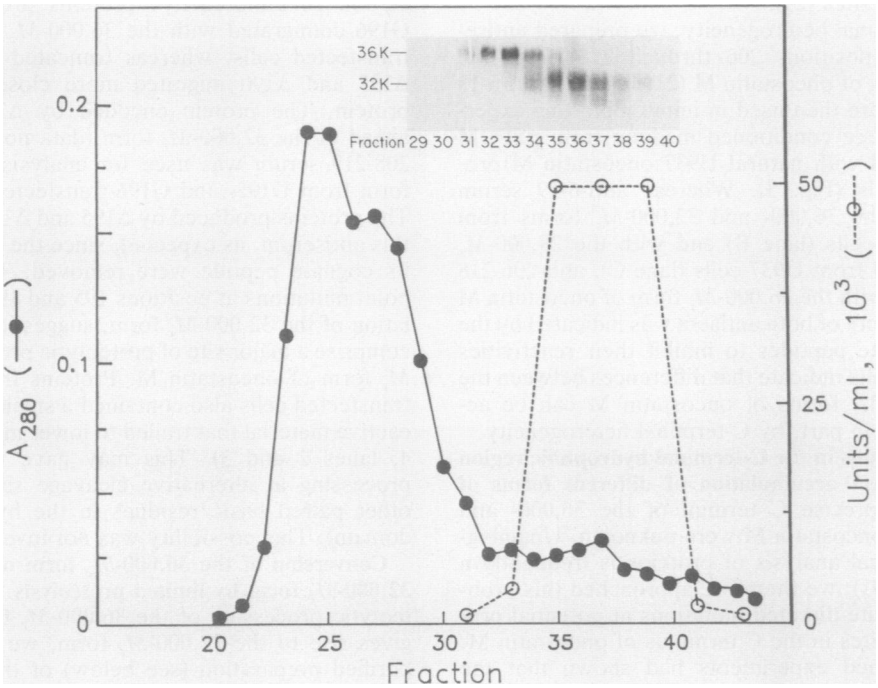


FIG. 6. Size fractionation reveals unequal growth-inhibitory activities of different forms of oncostatin M. COS cells were transfected with pβOM. Serum-free medium was collected, concentrated, and fractionated on a column of Bio-Gel P60 equilibrated with 1 N acetic acid as described previously (19). Fractions were collected, and the $A_{280}$ was determined (●). Samples were concentrated by evaporative centrifugation and analyzed for GIA activity (○). (Inset) The indicated fractions were analyzed by immunoblotting analysis with anti-6-19 serum.

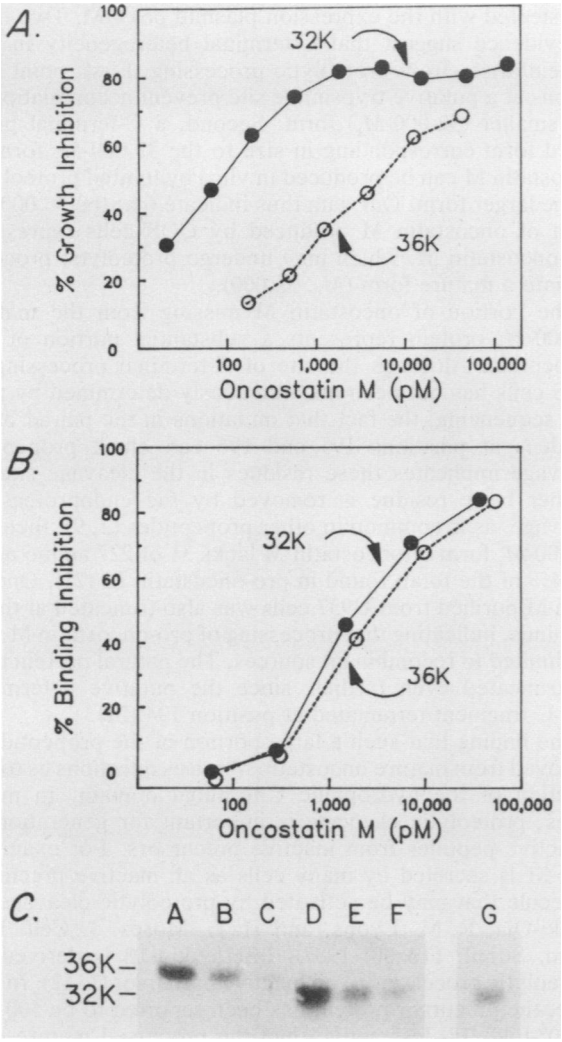


FIG. 7. Partially purified 36,000- and 32,000-$M_r$ forms of oncostatin M have different biological activities. The 36,000- and 32,000-$M_r$ forms of oncostatin M from pSPOM-transfected cells were partially purified as described in Materials and Methods. Pooled fractions containing the indicated forms of oncostatin M were tested for GIA (A) or RRA (B). (C) Quantitation of oncostatin M concentrations by immunoblotting analysis. Serial twofold dilutions of the 36,000-$M_r$ form of oncostatin M (lanes A through C, beginning with a volume of 15 μl) or 32,000-$M_r$ oncostatin M (lanes D through F, beginning with 4 μl) were analyzed by immunoblotting analysis. Relative amounts of oncostatin M in the indicated preparations were measured by reflective densitometry and converted to concentrations by comparison with reflectance of a standard amount (750 ng) of purified oncostatin M (lane G). The results of this analysis indicated that oncostatin M concentrations in the preparations of the 36,000- and 32,000-$M_r$ forms were 115 and 441 ng/ml, respectively.

purified 36,000-$M_r$ form had ~60-fold less GIA activity than the 32,000-$M_r$ form (half-maximal activities at 5,000 and 80 pM, respectively). In contrast, both forms had equivalent RRA activities (half-maximal activity at ~3,200 pM; Fig. 7B).

The experiment shown in Fig. 7 was repeated on a second preparation of the 36,000-$M_r$ form, which was purified by only a single cycle of size fractionation and consequently contained detectable amounts of the 32,000-$M_r$ form, as judged by immunoblotting analysis (data not shown). In this case, results were obtained that were qualitatively similar to those shown in Fig. 7, but the 36,000-$M_r$ form had approximately fivefold lower GIA activity and twofold higher RRA activity than did the 32,000-$M_r$ form. Thus, although the degree of reduction varied between preparations, the specific GIA activity of the 36,000-$M_r$ form was consistently less than that of the 32,000-$M_r$ form. In different experiments, the ratio of GIA to RRA activities for the 36,000-$M_r$ form of oncostatin M was also consistently less than that of the 32,000-$M_r$ form (0.4 and 0.7 for the 36,000-$M_r$ form and 2.4 and 21 for the 32,000-$M_r$ form), indicating that variation between preparations was not due to inaccuracies in measuring oncostatin M concentrations. Although we do not completely understand the variation in GIA activity between preparations, it may be partly explained by cross-contamination of the 36,000-$M_r$ form with the more active 32,000-$M_r$ form.

TABLE 1. Mutant forms of oncostatin M have different relative growth-inhibitory and binding activities[a]

| Plasmid | Activity (U/ng) | | GIA/RRA activity ratio |
|---|---|---|---|
| | GIA | RRA | |
| pSPOM | 27.2 | 6.1 | 4.5 |
| Δ195 | 74.3 | 3.8 | 19.5 |
| Δ190 | 21.7 | 1.0 | 22.5 |
| Δ182 | 0.06 | <0.06 | NA |
| G196 | 8.0 | 13.5 | 0.6 |
| G195 | 8.8 | 9.3 | 0.9 |

[a] Untreated serum-free media from COS cells transfected with the indicated plasmids were tested for GIA and RRA activities as described in Materials and Methods. Oncostatin M concentrations were determined by quantitative immunoblotting as described in Materials and Methods; these ranged from 0.4 to 1 μg/ml. Values indicate specific activities. NA, Not applicable.

**Mutant 36,000-$M_r$ forms of oncostatin M also have reduced growth-inhibitory activity.** Repeated attempts to purify the 36,000-$M_r$ form to homogeneity were unsuccessful due to the spontaneous accumulation of the 32,000-$M_r$ form during purification as the result of proteolytic degradation. Because of this difficulty, we sought to determine whether nonprocessed mutant forms of oncostatin M (G195 and G196) could be used as alternative sources for purification of a 36,000-$M_r$ form of oncostatin M.

We first compared biological activities of G195 and G196 with those of mutant proteins corresponding closely in size to the 32,000-$M_r$ form of oncostatin M (Δ190 and Δ182). For this experiment, untreated serum-free conditioned media from transfected cells were tested for GIA and RRA activities. Oncostatin M concentrations were determined by quantitative immunoblotting as described in Materials and Methods. Δ190 and Δ195 had higher GIA but lower RRA activities than G195 and G196 (Table 1). Ratios of GIA to RRA activities for Δ190 and Δ195 were 10- to 20-fold higher than those of G195 and G196. Medium from cells transfected with mutant Δ182 gave no significant activity in either assay, indicating that a portion of the C-terminal region from residues 182 through 190 was essential for both growth-inhibitory and binding activities. Medium from pSPOM-transfected cells gave intermediate activity ratios, consistent with the presence in this sample of two forms of oncostatin M having different activities. These observations indicate that mutant unprocessed forms of oncostatin M (G195 and G196) have less GIA activity than do the truncated 32,000-$M_r$ forms (Δ182 and Δ190). Furthermore, the extent of reduction in GIA activities was comparable to that of partially purified wild-type 36,000-$M_r$ form (Fig. 7), indicating

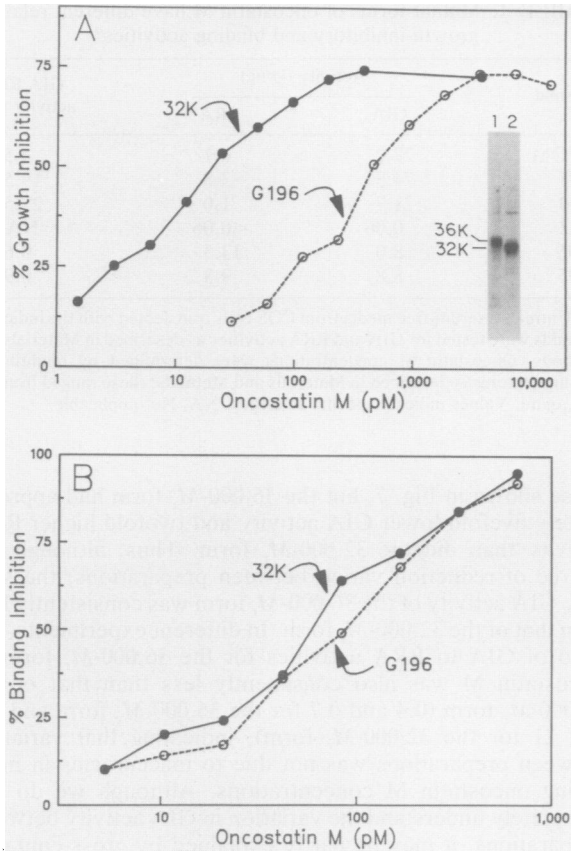


FIG. 8. Purified 32,000-$M_r$ oncostatin M and the mutant protein, G196, have different biological activities. The 32,000-$M_r$ form of oncostatin M (36K) and the G196 proteins were purified as described in Materials and Methods. Protein concentrations of the purified preparations were determined by amino acid analysis. The indicated amounts of protein were analyzed for GIA (A) or RRA (B) activity as described in Materials and Methods. (Inset, panel A) Samples of the purified preparations (0.75 μg) were analyzed by SDS-PAGE and Coomassie blue staining.

that mutations of the paired basic residues did not greatly reduce GIA activity.

The mutant protein from G-196-transfected cells was then purified to homogeneity and compared with the 32,000-$M_r$ form of oncostatin M purified from pSPOM-transfected cells (an SDS-PAGE gel showing purity of the preparations is shown in the inset of Fig. 8A). The purified G196 protein has less GIA activity than did the 32,000-$M_r$ form of oncostatin M (half-maximal activities at 150 and 22 pM, respectively) (Fig. 8A). In three separate experiments with these purified preparations, the differences in growth-inhibitory activities were 9-fold, 7-fold, and 5-fold (mean ± standard deviation, 7- ± 2-fold). In contrast, RRA activities of the two purified proteins were indistinguishable (half-maximal activities of approximately 100 pM; Fig. 8B). In the RRA assay, three separate experiments showed that G196 had approximately 1.1-, 1.1-, and 2-fold greater (1.3- ± 0.3-fold) RRA activity than did the 32,000-$M_r$ form of oncostatin M. Thus, purified G196 bound to the oncostatin M receptor equally well as purified 32,000-$M_r$ form but had less GIA activity.

## DISCUSSION

We have shown that two forms of the cytokine oncostatin M, differing in their C-termini, are produced by COS cells transfected with the expression plasmid pSPOM. Two lines of evidence suggest that C-terminal heterogeneity in this system arises from proteolytic processing. First, point mutations at a putative trypsinlike site prevent accumulation of the smaller (32,000-$M_r$) form, Second, a C-terminal processed form corresponding in size to the 32,000-$M_r$ form of oncostatin M can be produced in vitro by limited proteolysis of the larger form. Our data thus indicate that the 36,000-$M_r$ form of oncostatin M produced by COS cells represents pro-oncostatin M, which may undergo proteolytic processing into a mature form ($M_r$, 32,000).

The portion of oncostatin M missing from the mature 32,000-$M_r$ protein represents a substantial portion of the propeptide. Although the site of C-terminal processing in COS cells has not been unambiguously determined by protein sequencing, the fact that mutations in the paired basic residues at positions 195 and 196 can block propeptide cleavage implicates these residues in the cleavage site. If neither basic residue is removed by the endoprotease(s) cleavage, as is common in other propeptides (2, 9), then the 32,000-$M_r$ form of oncostatin M lacks 31 of 227 amino acids (~14% of the total) found in pro-oncostatin M (21). Oncostatin M purified from U937 cells was also truncated at the C terminus, indicating that processing of pro-oncostatin M was not limited to recombinant sources. The natural protein may be truncated even further, since the putative C-terminal Lys-C fragment terminated at position 194 (21).

The finding that such a large portion of the propeptide is removed from mature oncostatin M raises questions as to the function of the hydrophilic C-terminal domain. In many cases, proteolytic cleavage is important for generation of bioactive peptides from inactive precursors. For example, TGFβ1 is secreted by many cells as an inactive precursor molecule that may be activated by proteolytic cleavage (J. Keski-Oja, R. M. Lyons, and H. L. Moses, J. Cell. Biochem. Suppl. **11A**:60, 1987). Interleukin-1β is derived by proteolytic processing of an inactive precursor (1, 12). In this case, the precursor protein has been reported to be 500- (1) to $10^6$-fold (12) less active than the processed mature protein.

In other cases, the function of propeptide processing is unclear, since the propeptide is biologically active. For example, the membrane-bound precursor for transforming growth factor α was shown to have biological activity (32), although in this case a quantitative determination of relative activities of the propeptide and mature product was not made. Tumor necrosis factor is also derived from a membrane-bound precursor, which has been reported to be biologically active (15). In this case, the apparently active membrane-bound form may be released from the membrane by alternative cleavage as an inactive propeptide (6).

The pro-oncostatin M protein was 5- to 60-fold less active in the GIA than mature oncostatin M but was equally active in the RRA. Likewise, the nonprocessed mutant protein G196 was 6- to 20-fold less active in the GIA but equally active in the RRA. Thus, pro-oncostatin M is not activated by proteolytic cleavage to the same extent as is true for some propeptides (i.e., pro-interleukin-1β), but clearly has different biological activities than mature 32,000-$M_r$ oncostatin M. Proteolytic cleavage of the oncostatin M propeptide may therefore play a role in regulating the activities of this cytokine. It is possible that the difference in GIA activities of these forms may reflect a significant difference in as yet undiscovered in vivo activities of oncostatin M.

Another interesting possibility is that proteolytic processing of pro-oncostatin M may release biologically active

peptide(s) from the C-terminal domain. Although we do not currently know what the function of these peptide(s) might be, this type of processing, whereby small bioactive peptides are released from a much larger precursor, is common with neuropeptides (20).

It is not known at present whether processing of pro-oncostatin M occurs intra- or extracellularly. Many propeptides are processed intracellularly, beginning in the Golgi and continuing into the secretory vesicles (20, 28), but extracellular processing of propeptides is also common (e.g., the fibrinolytic and complement systems). In attempts to determine where oncostatin M was processed, we performed pulse-chase experiments on metabolically labeled COS cells transfected with pSPOM (data not shown). Preliminary experiments showed that the 36,000-$M_r$ form is detectable in the medium before the 32,000-$M_r$ form but have not allowed establishment of an unequivocal precursor-product relationship between these forms (data not shown).

The molecular basis for the difference in GIA activities of these two forms of oncostatin M is not presently known but apparently does not involve differences in receptor binding. This is shown by the experiment presented in Fig. 7, which demonstrates that both forms of oncostatin M compete equally well for binding of $^{125}$I-labeled oncostatin M. When data from binding inhibition curves for G196 and the 32,000-$M_r$ form of oncostatin M were plotted by the method of Scatchard (24), identical curvilinear plots were obtained (data not shown), suggesting that both forms bind equally well to high- and low-affinity oncostatin M-binding sites identified previously (18).

The finding that different forms of oncostatin M have similar receptor-binding properties but different GIA activities leads to the conclusion that receptor binding per se is not sufficient to initiate the growth-inhibitory activity of oncostatin M. This conclusion is also supported by results of previous studies, showing the presence of $^{125}$I-labeled oncostatin M-binding sites on cells that did not show a detectable biological response to oncostatin M (Horn et al., in press). Taken together, the results of the present and previous studies imply that intracellular signal(s), which under some circumstances may be generated as a consequence of receptor binding, are important in generating the growth-inhibitory activity of oncostatin M. The 36,000- and 32,000-$M_r$ forms of oncostatin M may therefore differ in their abilities to generate important intracellular signal(s).

It will be interesting to determine how cells that naturally produce oncostatin M regulate the relative levels of pro-oncostatin M and mature forms. The 36,000-$M_r$ pro-oncostatin M was not found in preparations purified from U937 cells (Fig. 3) or from activated T cells (data not shown). However, any pro-oncostatin M produced and secreted by these cells may have been discarded during purification due to reduced GIA activity or lost due to lability of pro-oncostatin M during purification. It therefore is possible that cells that naturally produce oncostatin M also secrete pro-oncostatin M. The role of proteolytic processing of pro-oncostatin M on the regulation of biological activities of this cytokine will be the subject of future studies.

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