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# Recombinant Expression, Biochemical Characterization, and Biological Activities of the Human MGSA/*gro* Protein†

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ABSTRACT: Melanoma growth stimulatory activity (MGSA) is a mitogenic protein secreted by Hs294T melanoma cells that corresponds to the polypeptide encoded by the human *gro* gene. The MGSA/*gro* cDNA has been expressed in mammalian cells and the secreted recombinant factor has been purified. Biochemical and biological characterization shows that the recombinant protein is identical with the natural protein and is devoid of posttranslational glycosylation, sulfation, and phosphorylation. The two C-terminal amino acids are proteolytically removed from the mature recombinant MGSA, indicating a length of 71 instead of the predicted 73 amino acids. The recombinant MGSA is mitogenically active on the Hs294T melanoma cells. The purified MGSA competes with interleukin 8 for binding to neutrophil receptors and exhibits neutrophil chemotactic activity equivalent to that of interleukin 8.

A variety of factors modulate the proliferation of mammalian cells in vitro and in vivo. These growth regulators can either stimulate or inhibit cellular proliferation depending on the nature of the factor, the cell type, and the physiological circumstances. The biological and structural studies on growth factors have largely concentrated on a limited set of growth factors, which by now have been relatively well-defined. However, there are still a variety of growth regulators that are less well-defined, often due to a lack of purified protein. One of these factors is known as "melanoma growth stimulatory activity" (MGSA). MGSA was initially identified as a factor secreted by the human melanoma cell line Hs294T with the ability to mitogenically stimulate these same melanoma cells (Richmond et al., 1982, 1983, 1985; Richmond & Thomas, 1986). Recent isolation and characterization of purified protein (Thomas & Richmond, 1988) and cDNAs (Richmond et al., 1988) have revealed that human MGSA is a single-chain polypeptide with a predicted length of 73 amino acids that is proteolytically derived from a 107 residue long precursor. The determination of the derived polypeptide sequence also established that human MGSA is identical with the gene product of the human *gro* gene. The cDNAs for *gro* were isolated and sequenced by Anisowicz et al. (1987), who proposed that the expression of this gene was growth-related. However, they did not define a specific activity or function for the gene product. Northern hybridization has established that the MGSA/*gro* gene is expressed by a variety of normal and transformed cells from different origins, such as fibroblasts, melanoma cells, epithelial cells, and endothelial cells (Anisowicz et al., 1987; Bordoni et al., 1989; Richmond et al.,

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1988; Wen et al., 1989). Its expression is thus certainly not restricted to melanoma cells, clearly suggesting that the activities of MGSA may be exerted on various target cell populations.

The deduced polypeptide sequence of human MGSA reveals that this factor is structurally related to various other polypeptides with β-thromboglobulin and platelet factor 4 as prototypes. This family also includes various other polypeptides, such as interleukin 8, γ-IP10, RANTES, and LD-78, which have as yet poorly defined activities and functions (Anisowicz et al., 1987; Bedard et al., 1987; Begg et al., 1978; Davatelis et al., 1988; Furata et al., 1989; Holt et al., 1986; Matsushima et al., 1988; Luster et al., 1985; Obaru et al., 1986, 1989; Oquendo et al., 1989; Poncz et al., 1987; Richmond et al., 1988; Schall et al., 1988; Schmid & Weissmann, 1987; Schroder et al., 1989; Deuel et al., 1981; Sugano et al., 1987; Walz et al., 1987; Westwick et al., 1989). All of these molecules are 8–10-kDa basic heparin-binding proteins. Among the functions associated with members of the β-thromboglobulin family are chemotaxis (Davatelis et al., 1988; Larsen et al., 1989; Schmid & Weissman, 1987; Tanaka et al., 1988; Wolpe et al., 1988), growth regulation (Richmond & Thomas, 1986; Richmond et al., 1988; Thomas & Richmond, 1988), stimulation of glycosaminoglycan synthesis (Castor et al., 1979), and mediation of the inflammatory response (Brown et al., 1989; Peveri et al., 1988). On the basis of their cellular sources and the conditions which induce the expression of the genes for these family members, it can be suggested that they may play a role in inflammation and in cellular proliferation. More specifically, we and others have shown that MGSA/*gro* expression is induced following treatment of cells with several factors such as the phorbol ester TPA, platelet-derived growth factor, thrombin, MGSA itself, and the inflammatory mediators interleukin 1, tumor necrosis factor, and lipopolysaccharides (Anisowicz et al., 1987, 1988; Bordoni et al., 1989; Richmond et al., 1988; Wen et al., 1989). Endothelial cells that play a crucial role in the immune defense mechanism and in inflammation secrete relatively high levels of the MGSA/*gro* polypeptide following exposure to these factors (Wen et al., 1989). However, it is obvious that the functions and the mechanism of action of MGSA cannot be defined in the absence of sufficient quantities of the purified protein. MGSA has as yet been purified only from the Hs294T cells (Thomas & Richmond, 1988), a process that is labor-intensive and cumbersome and results in relatively small amounts of the protein.

We herein report the establishment of stable mammalian cell lines that secrete high levels of human MGSA due to the introduction of an MGSA expression vector. Using an antiserum raised against a C-terminal peptide of MGSA, we were able to immunoprecipitate and detect the recombinant factor. Biochemical characterization established that the recombinant and the natural proteins have an identical size and N-terminus and lack detectable glycosylation, sulfation, and phosphorylation of the protein. Mass determination indicates that the last two amino acids of MGSA predicted by the cDNA sequence are proteolytically removed from the secreted polypeptide. Purified recombinant MGSA is mitogenically active on melanoma cells. It competes with interleukin 8 for binding to neutrophil cell-surface receptors and exhibits neutrophil chemotactic activity equivalent to interleukin 8.

## Materials and Methods

*Cell Culture and Transfections.* Chinese hamster ovary (CHO) cells deficient in the synthesis of dihydrofolate reductase (dhfr) (Urlaub & Chasin, 1980) were propagated in complete medium, i.e., Ham's F12 and Dulbecco's MEM (1:1), supplemented with 10% fetal bovine serum (FBS). Penicillin and streptomycin were included at 100 units/mL and 100 μg/mL, respectively. Transfections of plasmids into the mammalian cells were done essentially as described (Wigler et al., 1979). The dhfr-deficient CHO cells were cotransfected with 10 μg of pCMV-M23 and 200 ng of pSV-dhfr plasmid DNA as a calcium phosphate coprecipitate. Transfected CHO cells containing the dhfr gene were selected in complete medium lacking glycine, hypoxanthine, and thymidine and supplemented with 10% dialyzed FBS.

The human melanoma cell line Hs294T was obtained from the ATCC and grown in Ham's F10 medium supplemented with 10% FBS as described (Richmond et al., 1985).

*Plasmid Construction.* The expression vector pCMV-M23 designed for expression of MGSA in mammalian cells was derived from the plasmid pRK5 (Wood et al., 1988). Starting from the *Cla*I site and proceeding clockwise, there is first a segment of the MGSA cDNA (nucleotides 1–426; Richmond et al., 1988) that encodes the entire coding sequence and the proximal 89 nucleotides of the 3′ untranslated sequence down to the *Pst*I site. Following this fragment is the segment of SV40 DNA (originally a *Kpn*I–*Hin*dIII fragment; Fiers et al., 1978) that comprises the transcriptional termination and polyadenylation sites as well as the SV40 origin of replication and the early and late promoters. Next there is the *Sal*–*Eco*RI fragment of pML-1 that contains the bacterial origin of replication and the β-lactamase gene. Finally, the cytomegalovirus promoter and enhancer sequences precede the MGSA sequence and are responsible for the transcription of the MGSA cDNA. However, there is a small intron that contains the sp6 promoter between the initiation site of transcription and the unique *Cla*I site. This promoter is oriented such that it can direct the transcription of the MGSA cDNA in vitro. The plasmid pSV-dhfr contains a dihydrofolate reductase cDNA under transcriptional control of the SV40 early promoter and is very similar to plasmid pFD11 (Simonsen et al., 1983).

*Coupled in Vitro Transcription–Translation.* The MGSA cDNA in pCMV-M23 was transcribed in vitro from the bacteriophage sp6 promoter according to a modified protocol of Melton et al. (1984). The plasmid DNA was linearized with the *Hpa*I restriction endonuclease and was transcribed at a concentration of 50 μg/mL in a 100-μL reaction mixture containing 20 μL of 5× transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 20 mM spermidine), 10 μL of dithiothreitol at 100 mM, 20 μL of ribonucleotides (2.5 mM each of rATP, rCTP, rUTP, and rGTP), 41 μL of $H_2O$, and 1 μL of sp6 RNA polymerase. Transcription proceeded for 2 h at 37 °C and was stopped by chilling the reaction mixture on ice. Three-microliter aliquots of the transcription mixture were translated in a rabbit reticulocyte cell-free translation system in the absence or presence of canine microsomal membranes according to the manufacturer's protocol (Promega Biotec, Madison, WI). Following the incubation, SDS was added to a final concentration of 0.1% and the samples were treated for immunoprecipitation as described below.

*Metabolic $^{35}S$ and $^{32}P$ Labeling.* Cells were grown to about 70% confluency in six-well plates. The monolayers were washed twice with PBS and once with minimal medium and then incubated overnight at 37 °C in 1.5 mL of serum-free medium lacking cysteine and methionine or phosphate or sulfate (Gibco Laboratories). The media were then supplemented with 100 μCi/mL L-[$^{35}$S]cysteine and L-[$^{35}$S]-methionine or [$^{32}$P]$H_3PO_4$ or [$^{35}$S]$H_2SO_4$. After the overnight incubation the conditioned medium was collected and clarified

by centrifugation, and the protease inhibitor PMSF was added to the medium to a 1 mM final concentration.

*Immunoprecipitations Using an MGSA-Specific Antiserum.* An antiserum was raised in rabbits with an MGSA-specific oligopeptide as immunogen. The oligopeptide started with an N-terminal cysteine followed by the MGSA C-terminal sequence IEKMLNSDKSN and was conjugated to soybean trypsin inhibitor via a thioester linkage to the terminal cysteine. The characterization of the antibody has been previously described (Wen et al., 1989). Immunoprecipitations were carried out as follows. The samples were pretreated with 10 μL of normal rabbit serum and 20 μL of a 1:1 suspension of protein A–Sepharose at 4 °C for 1 h. The protein A–Sepharose was removed by centrifugation, and the pretreated sample was reacted overnight at 4 °C with the rabbit antiserum at a 1:100 dilution in a 500-μL reaction mixture to which 50 μL of protein A–Sepharose was added. The protein A–Sepharose beads were then pelleted and washed three times in 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 5 mM EDTA, and 10 units of Trasylol/mL. The washed beads were heated with 100 μL of SDS gel electrophoresis loading buffer at 100 °C for 5 min. The beads were removed by centrifugation, and the supernatant was loaded on a 16.5% Tricine–SDS–polyacrylamide gel (Schagger & Von Jagow, 1987).

*O-Glycanase Digestion.* Immunoprecipitated MGSA protein was eluted from protein A–Sepharose beads by boiling the samples for 5 min in 60 μL of 0.15% SDS. The protein sample in 50 μL, containing 1 mM calcium acetate, 10 mM D-galactono-γ-lactone, and 20 mM Tris–maleate, pH 6.0, was digested with neuraminidase at a final concentration of 1 unit/mL for 1 h at 37 °C, followed by *O*-glycanase (0.08 unit/mL) treatment for 6 h at 37 °C. After digestion, SDS electrophoresis sample buffer was added and the samples were boiled for 5 min prior to electrophoresis.

*Northern Hybridization.* Total cytoplasmic RNA was prepared as described (Rosenthal et al., 1986). Ten micrograms of RNA was denatured and loaded per lane in 1% agarose–formaldehyde gels (Maniatis et al., 1982). Northern hybridizations to the RNA transferred onto nitrocellulose filters were performed under highly stringent conditions (Derynck et al., 1988) by using the randomly $^{32}$P-labeled (Feinberg & Vogelstein, 1983) 700-bp *Eco*RI fragment of the MGSA cDNA (Richmond et al., 1988).

*Purification of Recombinant MGSA.* Five liters of serum-free conditioned medium from CHO clone 7 cells transfected with the expression plasmid pCMV-M23 was concentrated by lyophilization prior to dialysis against 0.17 M acetic acid. This concentrate was then subjected to Bio-Gel P-30 gel filtration chromatography. Fractions eluting between the ribonuclease A and insulin markers (13.7 and 6 kDa, respectively) were pooled, lyophilized, and subjected to heparin–Sepharose affinity chromatography as previously described (Thomas & Richmond, 1988). Fractions eluting from the heparin–Sepharose with 0.5 M NaCl were pooled, lyophilized, and dissolved in a solution containing 6% acetonitrile, 94% water, and 0.05% trifluoroacetic acid. Subsequent reversed-phase HPLC was on a Vydak Hi-Pore C18 column using a 60-min linear gradient from 6 to 60% acetonitrile. Aliquots of the 2-mL fractions were analyzed by SDS–polyacrylamide gel electrophoresis and by the MGSA bioassay to determine the purity and activity of the MGSA-containing fractions.

*N-Terminal Sequencing of Recombinant MGSA.* The recombinant MGSA preparations were run on a 15% SDS–polyacrylamide gel. The gels were then soaked in blotting buffer (25 mM Tris–base, 192 mM glycine, 15% methanol) for 15 min, and the separated proteins were then electrophoretically transferred for 2 h at 70 V onto PVDF (Immobilon, Millipore) sheets, which were presoaked in 100% methanol for 1 min and in blotting buffer for 15 min. The PVDF sheets were stained in 0.2% Coomassie blue in 50% methanol and 10% acetic acid for 1–2 min, destained, and air-dried. A 1-mm PVDF strip with the stained protein band was packed into the center of a sequencing column and sandwiched between 1 cm of controlled-pore glass and C18 reverse-phase packing (Baker). The protein was then sequenced by Edman degradation on a prototype liquid-phase sequencer as described in EP 257735. Reagents were purchased from Beckman (Quadrol and phenyl isothiocyanate), ABI (trifluoroacetic acid and 12.5% trimethylamine), or Bardick and Jackson (heptane, ethyl acetate, and butyl chloride).

*MGSA Bioassay.* MGSA bioassays were performed by measuring stimulation of [$^3$H]thymidine incorporation in serum-depleted low-density cultures of Hs294T human malignant melanoma cells essentially as described (Thomas & Richmond, 1988).

*Mass Spectrometry.* Mass spectra were recorded on a JEOL JMS-HX110HF/HX110HF mass spectrometer. Samples for mass analysis were prepared as described (Houtz et al., 1989; Robinson et al., 1989) by dissolving lyophilized HPLC fractions in 2–5 μL of 75% formic acid. A sample (0.5–1.0 μL; 100–500 pmol of peptide) of each fraction was added to 0.6 μL of *m*-nitrobenzoic acid on the sample probe. Peptides were sputtered from the liquid matrix into the gas phase for mass analysis largely in the form of $(M + H)^+$ ions by bombarding the sample matrix with $Cs^+$ ion projectiles (15 keV), and MSI was operated at 500 resolution.

*Chemical Cleavage and Peptide Purification.* Chemical cleavage with cyanogen bromide (CNBr) was performed by the addition of 300 μg of CNBr to 100 μL of 70% trifluoroacetic acid containing 1 μg of purified MGSA. The reaction vessel was flushed with nitrogen and then placed in the dark. The reaction was stopped after 24 h by the addition of 2 volumes of distilled water. The solvent was removed by lyophilization. The peptides were purified by HPLC using a Hewlett-Packard 1090A liquid chromatograph and an RP300 Aquapore column (Applied Biosystems). Solvents were 0.10% trifluoroacetic acid in water (solution A) and acetonitrile (solution B).

*$^{125}$I Interleukin 8 Competitive Binding Assay.* Recombinant interleukin 8 has been expressed in *Escherichia coli* and purified to homogeneity with a specific activity of $2 \times 10^6$ units/mg (Furata et al., 1989). Recombinant interleukin 8 was labeled with $^{125}$I according to the Bolton–Hunter method as previously reported (Falk et al., 1980; Samantha et al., 1989). The ability of recombinant MGSA to compete with $^{125}$I interleukin 8 ($1.0 \times 10^7$ cpm/μg) for binding to human neutrophils was examined as follows. In standard binding assays, $2 \times 10^6$ cells were incubated in duplicate with 4 ng of $^{125}$I interleukin 8 (40000 cpm) in RPMI 1640 medium containing 10 mg/mL BSA and 20 mM HEPES, pH 7.2, in a total volume of 200 μL at 4 °C essentially as described (Samantha et al., 1989).

*Chemotaxis Assay.* The neutrophil chemotaxis assay was performed as previously described (Falk et al., 1980; Larsen et al., 1989) except that recombinant MGSA was used.

## Results

*A Recombinant Expression Plasmid for MGSA.* In order to obtain a high level of expression of MGSA in recombinant mammalian cells, we constructed an expression plasmid pCMV-M23 that directs transcription of the MGSA coding

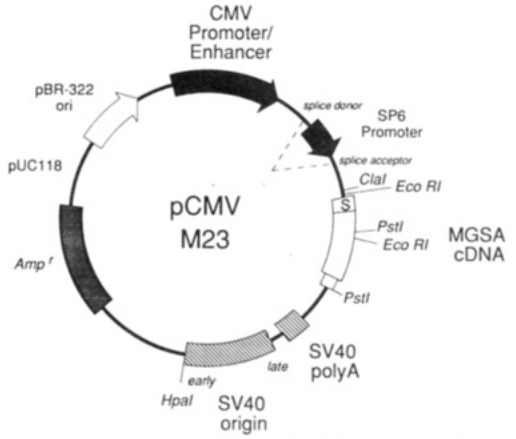


FIGURE 1: Schematic diagram of the pCMV-M23 expression plasmid. The cytomegalovirus promoter and the bacteriophage sp6 promoter are located upstream from the MGSA cDNA and are shown as black arrows. The S within the cDNA marks the signal peptide sequence. The SV40 polyadenylation segment and the origin of replication are boxed.

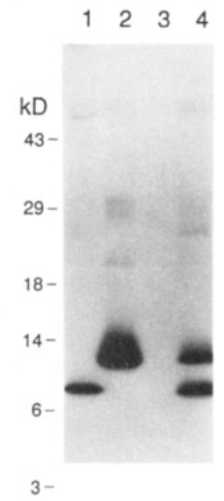


FIGURE 2: In vitro transcription–translation of MGSA cRNA derived from the plasmid pCMV-M23. Lane 1 shows as a reference the MGSA immunoprecipitated from conditioned medium of the Hs294T cells. Lanes 2 and 3 show the products of the translation in the absence of pancreatic microsomal membranes obtained after immunoprecipitation with the MGSA antibody. In lane 3 the immunoprecipitation was carried out after absorption of the antiserum with an excess of the MGSA C-terminal peptide to which the antiserum was raised. Lane 4 shows the translation products in the presence of the pancreatic microsomes obtained after immunoprecipitation with the MGSA antibody. The positions of the molecular weight markers are shown on the left.

sequence from the cytomegalovirus promoter. A schematic diagram of this plasmid is shown in Figure 1. The MGSA transcription unit contains the complete MGSA precursor coding sequence and the proximal 89 bp of the 3′ untranslated sequence. This short segment of the 3′ untranslated sequence did not contain any of the A-T-rich sequences with ATTTA as consensus sequence that are present in the 3′ untranslated sequence of the MGSA mRNA (Richmond et al., 1988). These sequences have been found in a variety of mRNAs often coding for cytokines and confer a high degree of instability to these mRNAs due to selective degradation (Caput et al., 1986; Shaw & Kamen, 1986). Thus these sequences were not incorporated in the transcription unit in order to avoid a fast degradation of the mRNA. The MGSA cDNA segment was followed by a DNA segment from SV40 that contains the sequences for transcriptional termination and polyadenylation of the SV40 late transcripts. The promoter element is separated from the MGSA transcription unit by a short intron that incorporates the prokaryotic sp6 promoter, allowing the generation of MGSA transcripts in vitro. This sp6 promoter is not functional in mammalian cells.

*In Vitro Transcription–Translation of the MGSA cDNA.* In vitro translation of individual mRNA species allows some structural characterization and evaluation of posttranslational processing of translated proteins. We generated complementary RNA by in vitro transcription of the MGSA cDNA from the sp6 promoter in the linearized pCMV-M23 plasmid. These transcripts were then translated in a cell-free rabbit reticulocyte lysate in the absence or presence of dog pancreas microsomes, and the translation products were immunoprecipitated with an MGSA-specific antibody (Wen et al., 1989). The deduced amino acid sequence of the 107 residue long MGSA precursor predicts that the mature 73 amino acid MGSA is generated following removal of the N-terminal sequence, which either represents solely the signal peptide involved in secretion or represents a signal peptide followed by a short propeptide as in the case of several hormone precursors (Amara et al., 1982; Gubler et al., 1982; Nakanishi et al., 1979; Noda et al., 1982) or of the EGF (Gray et al., 1983; Scott et al., 1983) or TGF-$\alpha$ precursor (Bringman et al., 1987). In vitro translation of the MGSA cRNA resulted in the synthesis of a single polypeptide that migrated as a band of 10–11 kDa in Tricine–SDS gels and could be immunoprecipitated with the antibody raised against the C-terminal peptide (Figure 2). Translation in the presence of dog pancreas microsomes generated two polypeptides that were recognized by the MGSA-specific antiserum. One of them was identical in size with the translation product in the absence of the microsomes, while the other one of about 8 kDa had the same mobility as the fully processed and secreted MGSA in conditioned Hs294T cell medium (Figure 2). The presence of these two bands strongly suggests that the microsomal processing was partial. The absence of an intermediate proteolytic cleavage product suggests that the upstream N-terminal sequence represents solely a signal peptide. It was occasionally observed that the in vitro translation resulted in multiple bands on the gel with molecular weight values corresponding to the MGSA monomer, dimer, and trimer, indicating the tendency of this factor to oligomerize (data not shown).

*Secretion of Recombinant MGSA by Transfected Cells.* The in vitro translation experiments described above established that the plasmid pCMV-M23 encoded a protein with the same size as authentic MGSA. This expression vector was then cotransfected with the plasmid pSV-dhfr containing a dihydrofolate reductase transcription unit into dhfr-deficient CHO cells. Forty-four transfected colonies that had acquired the dhfr-positive phenotype were screened by Northern hybridization for the expression of MGSA mRNA. We elected to screen by Northern hybridization rather than by MGSA bioassay due to possible interference from other secreted factors in this already tedious bioassay. Various MGSA mRNA levels were seen in about half of the transfected cell lines examined. As expected from the design of the expression plasmid, the MGSA mRNA in the transfected cells was about 0.9 kb long, compared to the 1.2-kb mRNA in the Hs294T melanoma cells (Figure 3). The transfected cell lines containing the highest levels of MGSA mRNA were examined for the secretion of recombinant MGSA by using immunoprecipitation of conditioned medium from cells metabolically labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine. As shown in Figure 4, with CHO clone 7 as an example, the transfected cells secrete a protein with an apparent molecular mass of 8 kDa in Tricine–SDS gels that can be specifically immuno-

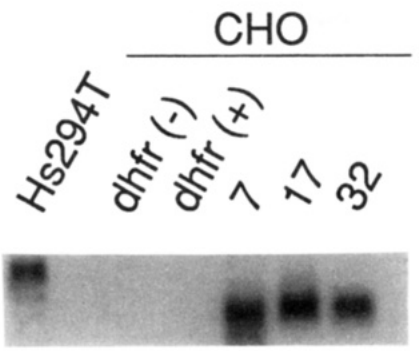


FIGURE 3: Northern hybridization of MGSA mRNA in transfected CHO cell lines. Total RNA was prepared from the Hs294T cells, nontransfected CHO cells, CHO cells transfected with the control plasmid pSV-dhfr, and CHO clones 7, 17, and 32 that were transfected with pCMV-M23. Ten micrograms of RNA was loaded per lane and hybridized.

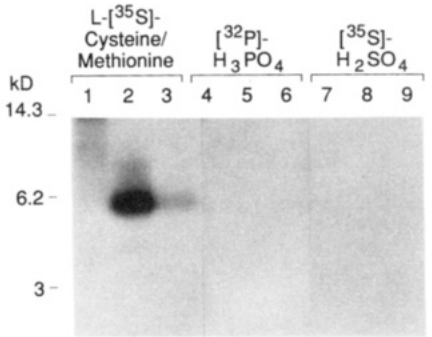


FIGURE 4: Immunoprecipitation of MGSA from serum-free conditioned medium of metabolically labeled CHO clone 7 cells and Hs294T cells. The isotope used for labeling was as shown above the lanes. Lanes 1, 4, and 7 show the results for a nontransfected CHO clone, lanes 2, 5, and 8 show the transfected CHO clone 7 cells, and lanes 3, 6, and 9 show the results for the Hs294T melanoma cells. The positions of the molecular weight markers are shown on the left.

precipitated by using the MGSA antibody. This MGSA was not present in the medium from parental nontransfected cells or from cells transfected with pSV-dhfr only. The secreted recombinant MGSA has the same mobility as the MGSA secreted by the Hs294T cells (Figure 4, lanes 2 and 3) or the processed MGSA translated in the reticulocyte lysate in the presence of microsomes (Figure 2).

*Biochemical Characterization of Recombinant MGSA.* In order to identify possible posttranslational modifications of both the natural and recombinant MGSA, the transfected CHO clone 7 cells and Hs294T cells were metabolically labeled overnight with 100 $\mu$Ci/mL [$^{35}$S]methionine and [$^{35}$S]cysteine or [$^{35}$S]$H_2SO_4$ or [$^{32}$P]$H_2PO_4$. Analysis of the immunoprecipitated medium showed that neither the natural nor the recombinant MGSA was labeled by $^{32}$P or $^{35}$S (Figure 4), indicating the lack of phosphorylation or sulfation of MGSA. Control experiments revealed that other proteins in the same cell extract or conditioned medium were accordingly labeled (data not shown). Since the predicted amino acid sequence does not contain a possible N-glycosylation site, we did not investigate the presence of N-linked carbohydrates. The possible presence of O-linked carbohydrates was evaluated by treatment of the $^{35}$S-labeled natural or recombinant MGSA with neuraminidase followed by *O*-glycanase digestion. Neither the natural nor the recombinant MGSA treated with neuraminidase plus *O*-glycanase showed a detectable shift in electrophoretic mobility when compared to the samples that were untreated or treated with neuraminidase only (Figure 5).

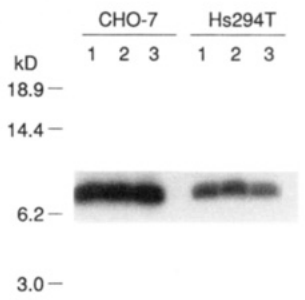


FIGURE 5: *O*-Glycanase treatment of immunoprecipitated $^{35}$S-labeled MGSA from the medium of the transfected CHO clone 7 cells and Hs294T cells. The samples were either not treated (lanes 1) or treated with neuraminidase (lanes 2) or with neuraminidase and *O*-glycanase (lanes 3).

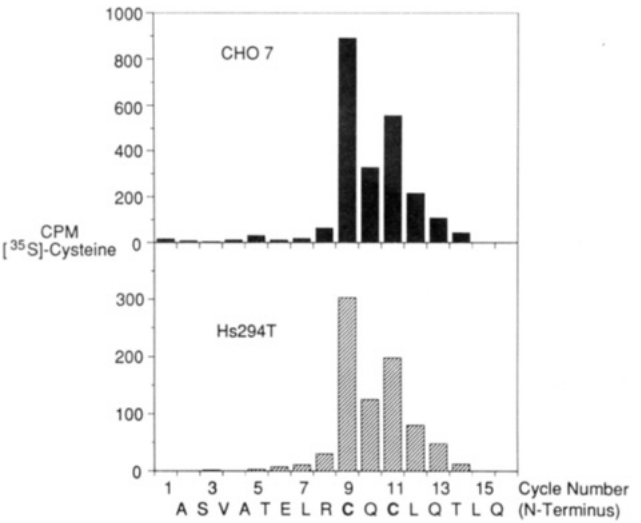


FIGURE 6: Isotope sequencing of natural and recombinant MGSA. The transfected CHO clone 7 cells and the Hs294T cells were metabolically labeled with L-[$^{35}$S]cysteine, immunoprecipitated, and separated on a 16.5% Tricine–SDS–polyacrylamide gel. The eluted 8-kDa MGSA was subjected to Edman degradation and analyzed over 15 cycles. The level of released radioactivity was determined for each cycle.

The N-terminus of the recombinant MGSA and thus the proteolytic cleavage site in the precursor were determined by direct sequencing of the [$^{35}$S]cysteine-labeled MGSA. The radiolabeled MGSA was immunoprecipitated from serum-free conditioned medium and isolated on a Tricine–SDS–polyacrylamide gel. The MGSA was eluted and subjected to 15 cycles of the Edman degradation reaction. The level of radioactivity released in each cycle was determined by liquid scintillation counting. This analysis showed that the peak radioactivity was released in the fractions corresponding to the 9th and 11th residues from the N-terminus (Figure 6). Comparison of these data with the experimentally determined N-terminus of the natural MGSA and with the polypeptide sequence for the precursor derived from the cDNA sequence indicates that the recombinant MGSA has the same N-terminus as the natural MGSA secreted by the melanoma cells (Figure 6).

*Purification of Recombinant MGSA.* Five liters of conditioned medium from the transfected CHO clone 7 cells was harvested and processed for purification of the recombinant MGSA. The medium was lyophilized, dialyzed against 0.17 M acetic acid, and subjected to sequential gel filtration on Bio-Gel P-30 and affinity chromatography on heparin–Sepharose. The fractions eluted from the heparin matrix with 0.5 M NaCl were then further purified by reversed-phase HPLC on a hydrophobic C18 matrix as described (Thomas

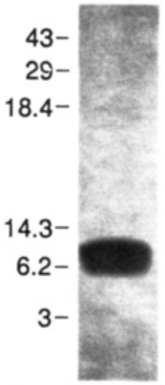


FIGURE 7: Purified recombinant MGSA. The MGSA was analyzed by SDS–polyacrylamide electrophoresis and stained with Coomassie blue. The positions of the molecular weight markers are shown on the left.

& Richmond, 1988). Throughout all steps, the purification of the recombinant MGSA was followed by analytical SDS gel electrophoresis and by the MGSA bioassay for mitogenicity on Hs294T melanoma cells. The purified MGSA eluted from the reversed-phase column at 35% acetonitrile and was homogeneously pure as assessed by gel electrophoresis (Figure 7). The MGSA has an apparent molecular mass of about 8 kDa when analyzed on a polyacrylamide–SDS gel. The N-terminal sequence of the purified protein established its identity as recombinant human MGSA and established again that its N-terminus is identical with the natural melanoma-derived MGSA (data not shown). The yield of the purified MGSA from the initial 5 L of medium was about 60 μg, based on the relative quantitation of the integrated HPLC peak in comparison with an insulin standard.

*Mass Determination of Recombinant MGSA.* A mass spectrum obtained on 1 μg of recombinant MGSA revealed an abundant protonated [$(M + H)^+$] polypeptide at $m/z$ 7661.0. This observed mass is in contrast to the calculated protonated molecular mass for recombinant MGSA ($m/z$ = 7862.2) based on the predicted sequence of mature MGSA. Since N-terminal sequence analysis has established the expected sequence, we suspected that a C-terminal proteolytic cleavage had occurred. Removal of the two C-terminal residues (Ser and Asn) would result in a predicted $(M + H)^+$ ion at $m/z$ 7661.1, which is in very close agreement with the observed $(M + H)^+$ ion at $m/z$ 7661.0, thus predicting that the C-terminal residue is a Lys. In addition to the major peak observed in the mass spectrum of recombinant MGSA, an ion at $m/z$ 7698.7 was also present, which on the basis of observations with other proteins presumably corresponds to a $Na^+$ adduct of the Met sulfoxide derivative of MGSA. The observed ion at $m/z$ 7698.7 is in close agreement with the predicted protonated molecular mass of 7698.2 for this chemical derivative. In order to confirm the structure of the C-terminus of mature MGSA, the protein was cleaved with CNBr. There is only one Met residue in the amino acid sequence for MGSA, which is close to the C-terminus (Richmond et al., 1988). The C-terminal peptide should have the sequence Leu-Asn-Ser-Asp-Lys, which upon ionization in the mass spectrometer would result in an $(M + H)^+$ ion at $m/z$ 576.2 if indeed the C-terminal Asn and Ser residues were removed. The small C-terminal peptide was purified by HPLC following CNBr treatment of the MGSA, and analysis of this peptide by mass spectrometry determined an abundant protonated polypeptide ion at $m/z$ 575.9, which is in close accordance with the predicted mass value. This analysis strengthens the conclusion that the two C-terminal amino acids of MGSA as predicted from the cDNA sequence have been proteolytically removed.

Table I: Mitogenic Effect of Recombinant MGSA on Hs294T Cells[a]

| concn (ng/mL) | [$^3$H]thymidine uptake as % of control (±SD) | cell no. as % of control (±SD) |
|---|---|---|
| 0.06 | 176 (±20) | 277 (±43) |
| 0.6 | 219 (±17) | 270 (±44) |
| 6 | 216 (±15) | 140 (±20) |
| 60 | 140 (±16) | nd |

[a] The values for [$^3$H]thymidine uptake and for the cell numbers were obtained in different experiments. nd, not determined. Each set of data corresponds to one experiment representative of eight experiments. Each data point is the average of triplicate values.

Table II: Chemotaxis Assay Using Human Neutrophils[a]

| concn | migrated cell no./high power field (40 × 10)[b] | chemotactic index |
|---|---|---|
| MGSA | | |
| 0 ng/mL | 5.8 | 1 |
| 0.05 ng/mL | 6.0 | 1.1 |
| 0.5 ng/mL | 17.5 | 3.0 |
| 5.0 ng/mL | 48.3 | 8.3 |
| 50.0 ng/mL | 101.8 | 17.5 |
| 500 ng/mL | 86.5 | 14.9 |
| fMet-Leu-Phe, $10^{-7}$ M | 123.0 | 21.2 |

[a] The chemotactic index equals the migrated cell number in the presence of chemoattractant divided by the cell number using the media only. [b] $n = 6$; mean value is shown.

*Mitogenic Activity of Purified Recombinant MGSA.* In order to assess whether the purified recombinant MGSA is biologically active, we determined if this protein had a mitogenic activity on the Hs294T cells. This activity originally led to the identification of MGSA (Richmond et al., 1982, 1983, 1985; Richmond & Thomas, 1986). The mitogenicity was determined by measuring the incorporation of [$^3$H]thymidine in these melanoma cells and by determining the cell numbers as previously described (Table I). The increase in DNA synthesis in these cells has previously been shown to correlate well with a proportional increase in cell number (Richmond et al., 1982, 1983, 1985; Richmond & Thomas, 1986). The purified recombinant MGSA was tested at several concentrations from 0.06 to 60.0 ng/mL, the increase in DNA synthesis was measured after 16 h, and the cell numbers were determined after 3–6 days of exposure to MGSA. The presence of recombinant MGSA resulted in increases in radiolabeled DNA and cell number (Table I). A decrease of mitogenic activity at higher concentrations, often observed with growth factors, is also apparent in the case of MGSA. Addition of bovine serum to a final concentration of 1% resulted in a 102% increase in [$^3$H]thymidine incorporation in a typical experiment (data not shown).

*Competition of MGSA with $^{125}$I Interleukin 8 in Neutrophil Binding Assay.* The structural similarity between MGSA and several other members of the β-thromboglobulin family that are chemotactic for neutrophils led us to examine the ability of MGSA to compete with $^{125}$I interleukin 8 for binding to neutrophils. $^{125}$I interleukin 8 specifically binds to human neutrophils, and 100 ng of unlabeled interleukin 8 displaces approximately 50% of the $^{125}$I-labeled interleukin 8 from these cells. MGSA competed with $^{125}$I interleukin 8 for receptor binding with 50% competition at 500 ng/mL. This corresponds to a 5-fold higher concentration than with interleukin 8 for a 50% inhibition of binding of $^{125}$I interleukin 8 to the cell-surface receptors (Figure 8).

*Neutrophil Chemotactic Activity of MGSA.* Since interleukin 8 shows in vitro chemotactic activity, we tested MGSA for its possible chemotactic activity for human neutrophils

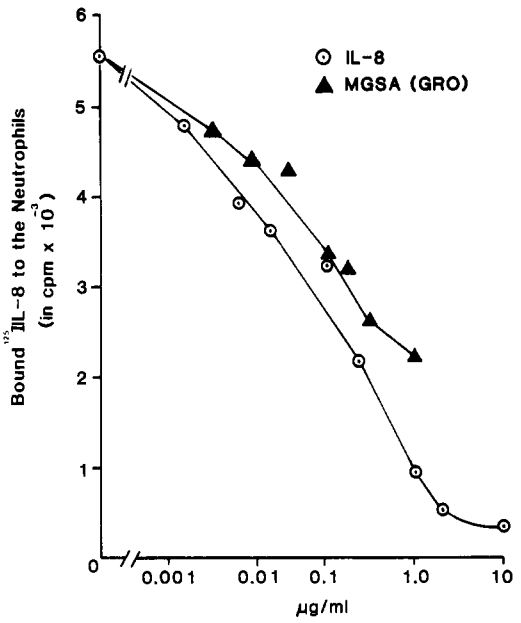


FIGURE 8: MGSA/interleukin 8 competitive binding curves. Human neutrophils ($2 \times 10^6$ cells) were suspended in binding medium and incubated with $^{125}$I interleukin 8 with or without varying concentrations of unlabeled MGSA or interleukin 8 for 2 h at 4 °C. Cells were washed once and pelleted through a sucrose cushion, and bound radioactivity was counted in a $\gamma$ counter.

(Table II). Concentrations as low as 0.5 ng/mL MGSA resulted in a chemotactic index of 3.0, while the peak response was at 50 ng/mL with a chemotactic index of 17.6. There was a decline in the chemotactic index when 500 ng/mL MGSA was used. For comparison, administration of fMet-Leu-Phe at $10^{-7}$ M resulted in an index of 21.2, comparable to the 50 ng/mL concentration of MGSA. Thus the dose-dependent potency of neutrophil chemotactic activity of MGSA is essentially equal to recombinant interleukin 8 (Larsen et al., 1989).

## Discussion

In this report we have documented the expression of recombinant human MGSA/*gro* and have characterized the purified growth factor. The established cDNA sequence has established that MGSA is encoded as a 107 amino acid precursor from which the N-terminal 34 amino acid sequence is removed in order to generate the mature growth factor polypeptide. Coupled in vitro transcription and translation show that the dog pancreas microsomes are able to remove the 34 amino acid segment. This proteolytic processing in vitro was only partial so that both the unprocessed protein and the proteolytically cleaved MGSA were detected. The lack of intermediate products suggests that the 34 amino acid sequence represents solely the signal sequence and is not the combination of a signal peptide and a prosequence. The N-terminus of the recombinant MGSA was established by sequence analysis of metabolically radiolabeled MGSA and by analysis of the purified protein. The N-terminal sequence was identical for both the recombinant factor and the MGSA secreted by the melanoma cells. Using metabolic labeling with $^{32}$P or $^{35}$S and enzymatic deglycosylation, we established that the mature MGSA does not undergo posttranslational modifications such as phosphorylation, sulfation, or glycosylation. Analysis by mass spectrometry, which results in very accurate mass determinations, indicated that the mass is lower than expected for the unmodified 73 amino acid polypeptide but corresponds exactly to the value for the polypeptide without the two C-terminal amino acids, predicting that a cleavage takes place at that position following the now C-terminal lysine. Proteolytic cleavage following a lysine residue has been described for several other polypeptides (Amara et al., 1982; Gubler et al., 1982; Nakanishi et al., 1979; Noda et al., 1982; Neurath et al., 1986) and may be due to the action of trypsin-like proteases (Graf & Kennessey, 1981; Neurath et al., 1986). The established mass value and the accuracy of the method used also preclude the presence of other posttranslational modifications of the MGSA polypeptide.

The purified recombinant MGSA stimulates the proliferation of the Hs294T melanoma cells. Stimulation of these cells is observed at the nanogram per milliliter level similar to those of other more established growth factors. The increase in DNA synthesis is only 2–3-fold, but it should be taken into account that the target cell used for this assay has a high endogenous level of MGSA synthesis (Richmond et al., 1982, 1983, 1985; Richmond & Thomas, 1986). There is currently only minimal knowledge about other cells that are responsive to the mitogenic effect of MGSA. It is possible that the major activity of MGSA/*gro* is not the stimulation of DNA synthesis but that the mitogenic effect of MGSA is only a delayed consequence of the stimulation of some other parameters.

MGSA and interleukin 8 are structurally related. It is thus not surprising that both factors interact with the same receptors and display similar biological activities as illustrated by their stimulation of the chemotactic activities of neutrophil cells. As both MGSA and interleukin 8 belong to a larger family of factors, it is likely that all of these related polypeptides can interact with the same receptors, albeit with differences in affinity, resulting in similar biological activities, such as chemotaxis. MGSA synthesis is induced by a variety of inflammatory mediators (Wen et al., 1989), and it has been shown that the murine KC mRNA, which may be a homologue of the human MGSA (Oquendo et al., 1989), is also induced following mitogenic stimulation with serum- or platelet-derived growth factor (Cochran et al., 1983). Exposure of cells to these various factors will thus result not only in the primarily induced physiological or behavioral changes but also in the release of chemotactic polypeptides, which in turn may act in an autocrine fashion on the same cells or in a paracrine way on surrounding cell populations.

By analogy with many other growth factors and cytokines, it is possible that MGSA exerts several other activities besides mitogenic and chemotactic effects. As the biological characterization of this factor has only been recently initiated, it is clear that further studies will have to be pursued in order to fully understand the effects of the secretion of this factor on the physiology of the target cell. The availability of recombinant MGSA/*gro*, which overcomes the labor-intensive generation of only small amounts of the natural molecule, should certainly facilitate these studies.

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# Effect of Increased Lipid Packing on the Surface Charge of Micelles and Membranes†

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ABSTRACT: We have investigated the responsiveness of micelle and bilayer surfaces to changes in bulk pH through titrations, and to changes in lipid packing through the application of high hydrostatic pressure using two fluorescent, pH-sensitive surface probes. In micelles, the surface is very sensitive to bulk pH while in phosphatidylcholine and phosphatidic acid bilayers the surface charge changed little through a large pH region. Application of pressure on micelles causes proton dissociation due to the volume reduction achieved from the contraction of water around the charges (electrostriction). However, in bilayers, the effect of electrostriction is greatly reduced, most likely due to the energy needed to expand and hydrate the surface. The sign and amount of change in dissociation the probe undergoes with pressure depend on the initial degree of probe dissociation, which is in turn dependent on the particular surface rather than the charge of the lipid head groups comprising the bilayer. This finding may limit the use of fluorescent probes to determine the exact surface potential. By assuming the change in $\Delta V$ for proton dissociation from the probe is constant for a given pH, we can calculate the changes in local pH that occur under pressure relative to a neutral or uncharged system. In doing so, we find that the local pH around the probe in bilayers changes very little ($\sim$0.1 pH unit or less) in the first 2000 bars. Also, if pressure data are coupled with titration curves, we can determine the change that the bulk pH must undergo to produce the observed change in dissociation seen under pressure. Here, we find that raising the pressure from 1 to 2000 bar has no effect on dioleoylphosphatidylcholine bilayers whereas raising the pressure of dioleoylphosphatidic acid bilayers is equivalent to decreasing the bulk pH from 7 to 3. Although this appears to be a large effect, the actual change in surface charge in this pH region is quite low.

The physical state of a membrane bilayer is the result of the particular interactions occurring on the surface and in the interior. Since lipid packing may regulate the aggregation and function of integral membrane proteins, it is important to understand the role lipid–lipid interactions play in determining membrane properties. While previous work has mainly focused on the influence of hydrocarbon packing on the phase and fluidity of bilayers, there is very little information about how packing modulates the interactions between surface groups (Cevc, 1987). The goal of this study is to determine the effect of lipid packing on the surface charge.

In vivo, the ambient temperature and pressure surrounding a cell are relatively constant, and lipid packing is controlled by careful regulation of the composition. Here, packing will be varied by hydrostatic pressure since altering the membrane composition makes analysis on the chemical level difficult and varying the temperature produces changes that are due to a combination of both volume and kinetic energy. Many membrane systems have been investigated using high pressure [see Heremans (1982)], and it has been found that pressure increases the gel-to-liquid-crystal-phase transition by approximately 20 °C/kbar (1 bar = 1.013 atm). The effect of high hydrostatic pressure on bilayers surface has not yet been characterized (MacDonald, 1984).

While many naturally occurring lipid head groups have no net charge, many are negatively charged and have a tendency to repel each other. This repulsion could be partially relieved by hydration, counterion binding, and, where possible, hydrogen bonding. We are specifically interested in determining whether increasing chain packing through high pressure could destabilize the bilayer due to increased repulsion between head groups. To accomplish this, we will use fluorescent pH indicators that are sensitive to changes in surface pH and thus charge. Previously, pH probes have been used extensively to characterize the surface potential of various membranes (Fromherz, 1973; Fromherz & Masters, 1974). Using one of these probes embedded in phosphatidylethanolamine (PE)[1]

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[1] Abbreviations: PE, phosphatidylethanolamine; DECNA, 6-decanoylnaphthol; HEXCO, 3-hexadecanoylcoumarin; OG, *n*-octyl glucoside; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; DMPA, dimyristoylphosphatidic acid; DMPS, dimyristoylphosphatidylserine; DOPG, dioleoylphosphatidylglycerol.

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