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IDENTIFICATION OF AN ANTIGENIC DOMAIN NEAR THE C-TERMINUS OF HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND ITS SPATIAL LOCALIZATION

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SUMMARY

The aim of this study was to map an epitope on human granulocyte-macrophage colony-stimulating factor (hGM-CSF) at its C-terminus, a region whose integrity is fundamental to maintaining the normal function of this molecule. Residues including the fourth α-helix (D, 103-116) were analyzed for their role in the interaction with antibodies (Abs) raised against the protein. Five peptides homologous to different segments of the C-terminus of hGM-CSF were synthesized. Peptide (102-121) included the same residues of the α-helix D and the next five aminoacids toward the C-terminus; peptide [E108A](102-121) introduced the mutation E108A in order to verify the role of acidic residues; peptide [C96A](93-110) encompassed the β-sheet 2 and half of the α-helix D; peptide [C121A](110-127) included the second half of the α-helix D and the C-terminus of hGM-CSF; peptide (13-31)-Gly-Pro-Gly-(103-116) included both the α-helices A and D connected by the tripeptide Gly-Pro-Gly, which allows the original antiparallel orientation of the two α-helices to be maintained. Both anti-protein and anti-peptide (102-121) Abs, capable of neutralizing the stimulatory activity of hGM-CSF in the bone marrow colony-forming assays, recognized a specific epitope in the C-terminus of hGM-CSF. Molecular modeling estimated the surface accessibility of hGM-CSF and the stability of the synthetic peptides in aqueous solution. Altogether, our results showed that the immunogenic region includes part of the α-helix D and the residues 116-120, which are external to this helix and particularly exposed on the protein surface, confirming the feasible participation of this region in antibody binding.

INTRODUCTION

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a pleiotropic growth factor essential for the survival, growth and differentiation of hematopoietic cells (1-5). It also affects the growth of a variety of cell types of non-hematopoietic origin, including placental trophoblasts, endothelial cells, dendritic antigen-presenting cells and osteoblastic cells (6-11). Furthermore, hGM-CSF acts as an autocrine-paracrine growth factor for tumor cell lines of different histogenesis, including myeloid leukemia, glioma, prostate, and epithelial neoplastic cells (6;12-20).

hGM-CSF is secreted as a 127-aminoacids (14.65 KDa) glycoprotein with a final molecular weight in the range of 18-25 KDa, depending on its glycosylation. Removal of oligosaccharides does not reduce the biological activity of hGM-CSF, confirming their implication only in the half-life prolongation of the protein and not in receptor binding (21).

Several different approaches have been taken to define the relationship between the structure and the functional areas of hGM-CSF. Previous studies using synthetic peptides and neutralizing antibodies, substitution and deletion mutants and interspecies chimeric polypeptides, have indicated several areas whose integrity is fundamental to maintaining the normal function of the growth factor (22-28).

The structure of hGM-CSF solved by x-ray crystallography (PDB 2GMF) shows its overall organization as an open bundle of four α-helices, named A (13-28), B (55-64), C (74-87) and D (103-116), combining with two anti-parallel β-sheets (39-43 and 98-102) (Figure 1A). The polypeptide tertiary structure is stabilized by two disulphide bridges (C54-C96 and C88-C121) (29;30). Similar structural features are shared by a group of related cytokines, growth factors, and

hormones (31). Biological responses stimulated by hGM-CSF occur following its binding to a specific heterodimeric surface membrane receptor present in sensitive target cells (32;33). This receptor consists of two different trans-membrane glycosylated polypeptide chains named GM-CSF.Rα and GM-CSF.Rβ . Both are members of the hemopoietin receptor superfamily (34-37).

GM-CSF.Rα binds specifically hGM-CSF with relatively low affinity (2-13 nM) but in the absence of the β-chain, no signal is transmitted to the cell. GM-CSF.Rβ, which is shared by GM-CSF, IL-3 and IL-5 (33;38;39), by itself shows no detectable binding to the ligand, but once the hGM-CSF/GM-CSF.Rα complex is formed, the β-chain likely interacts with this complex, resulting in a more stable tertiary structure, increasing the cytokine binding affinity (25-100 pM) and amplifying signal transduction (33;35-37;40;41).

Recombinant hGM-CSF (rhGM-CSF) is currently used in patients with acquired immunodeficiencies, myelodysplastic syndromes, and chemo- or radio-therapy-induced neutropenia (13;16;42;43). However, it has been found that a relatively high proportion of human sera and purified immunoglobulin (Ig) preparations collected from blood of normal healthy adults or from neonatal cord blood contains low levels of high-affinity anti-hGM-CSF antibodies (Abs) that are normally non-neutralizing (14;25;44). Naturally occurring anti-hGM-CSF Abs with blocking activity are sometimes present in pharmaceutically prepared human Igs, confirming the notion that these auto-Abs contribute to the immunomodulatory activities of these Ig preparations (14;44;45). Furthermore, after repetitive treatments with rhGM-CSF, patients with inflammatory disorders or neoplastic diseases may generate neutralizing anti-hGM-CSF auto-Abs capable of recognizing both natural (glycosylated) and unglycosylated hGM-CSF (15;17-20). Recently, it has been reported that patients with idiopathic pulmonary alveolar proteinosis (IPAP) with age may develop progressively increasing titers of neutralizing anti-hGM-CSF auto-Abs (46-48). Detailed

information on the immunodominant epitopes that may preferentially stimulate neutralizing anti-hGM-CSF auto-Ab production is needed for monitoring the most effective use of rhGM-CSF as a therapeutical agent.

Several distinct domains of the rhGM-CSF protein have been found to be immunogenic in animals as well as in human patients treated with the exogenous cytokine (24-26;28). It has been proposed that the two α-helices A and D, which are in close proximity due to the tertiary folding of the protein (29), are involved in the binding of hGM-CSF to the receptor and are critical for function (28). Both α-helices A and D stimulate the production of neutralizing Abs in rabbits, mice and humans treated with exogenous rhGM-CSF (24-26;48).

The aim of this study was to use synthetic peptides and anti-protein as well as anti-peptide (102-120) Abs in order to identify and map the position of critical aminoacid residues within the C-terminal region and to establish their relative contribution for Ab binding. Molecular modeling techniques have been used to ascertain the surface accessibility and stability of these above peptides in aqueous solution.

EXPERIMENTAL PROCEDURES

rhGM-CSF, synthetic peptides and protein conjugates

Highly purified recombinant human GM-CSF (rhGM-CSF), 127 aminoacid, with a molecular weight of 14,650 KDa and a specific activity of about 10,000 units/μg/ml in the human bone marrow colony-forming assays (49), was obtained through the courtesy of Dr. Federico Bertolero, from Farmitalia-Carlo Erba Biotechnology Facility (now Pharmacia & Upjohn, Nerviano, Milan, Italy).

Overlapping peptides encompassing the C-terminal region of the hGM-CSF polypeptide were synthesized by the continuous-flow solid-phase method on an automated peptide synthesizer using fluorenyl-methyloxy carbonyl (Fmoc) chemistry (Milligen 9050). Synthesis was carried out using the resin Novasyn TGR (Novabiochem) on a polystyrene-supported polyoxyethylene scaffold (PEG-PS), in order to obtain a C-terminal amide by means of acidic cleavage. Cleavage from the resin and side-chain deprotection was achieved by treatment of the dried peptide-resin with 10 ml of a mixture of trifluoroacetic acid (TFA)/thioanisole/ethanedithiol/water/phenol (82.5:5:2.5:5:5% v/v) for 1 h at room temperature. The crude peptide was precipitated with cold diethylether and lyophilized. The crude peptide was analyzed by high-performance liquid chromatography (HPLC, Beckman System Gold) under the following conditions: Vydac C_{18} column (0.46 x 15 cm^3); eluent A, 0.1% TFA/water; eluent B, 0.1% TFA/acetonitrile; gradient from 20% to 80% B over 20 min; flow, 1 ml/min; detection UV, 210 nm. The main peak was isolated by preparative HPLC under the following conditions: Vydac C_{18} column (2.2 x 25 cm^3); eluents A and B as indicated above; gradient from 15% to 40% B over 160 min; flow, 4 ml/min; detection, UV, 210 nm. The final, purified peptide exhibited the correct amino acid ratios of the acid hydrolysate and an HPLC purity

greater than 99%. The peptide was further characterized by mass spectrometry (ES-MS), yielding the correct molecular weight and sequence.

The sequence of the first synthetic peptide (102-121) included the same residues of the α-helix D (103-116) and the next five residues of hGM-CSF (Figure 1B). We introduced the mutation E108A to the same peptide, [E108A](102-121), in order to study the possible role of acidic residues. To complete the characterization of the putative epitope in the C-terminus of the hGM-CSF, we synthesized two other peptides: [C96A](93-110), which is analogous to the hGM-CSF region between the middle of the β-sheet 2 and the end of the α-helix D, and [C121A](110-127), which is analogous to the region between the middle of the α-helix D and the C-terminus of hGM-CSF (Figure 1B). To better understand a possible cooperation of α-helices A and D on the immunogenicity of hGM-CSF, particularly referring to the C-terminus region epitope, we synthesized a further peptide, (13-31)-Gly-Pro-Gly-(103-116), which includes the sequences of both the α-helices connected by the tripeptide Gly-Pro-Gly. The α-helix disruptor character of the linking tripeptide (50) allows the original antiparallel orientation of the two α-helices A and D to be maintained, in a conformation very similar to the crystal structure of hGM-CSF, as confirmed by NMR analysis (51).

Antibody production

Anti-rhGM-CSF polyclonal Ab production. Primary immunization of two outbred New Zealand White rabbits was done by means of subcutaneous foot-pad injection of 200 μg of purified rhGM-CSF in Freund's complete adjuvant (FCA; Sigma, St. Louis, MO, USA). After 15 days, each animal received an intramuscular boost with 200 μg of rhGM-CSF in FCA, and then at 2-week intervals, five intradermal injections in the dorsal skin with 50-100 μg of rhGM-CSF in Freund's

incomplete adjuvant (Sigma) per spot, until a clear Arthus reaction became visible. Each rabbit was bled 3 days after the last injection, and the serum was collected and stored in aliquots at –20°C until use. The two rabbit sera anti-rhGM-CSF obtained were labelled 813 and 814.

Anti-(102-121)-KLH polyclonal Ab production. The peptide 102-121 was conjugated to keyhole limpet hemocyanin (KLH) and to bovine serum albumin (BSA) with the linker maleimide (Imject Maleimide Activated Keyhole-Limpet Hemocyanin and Imject Maleimide Activated Bovine Serum Albumin, Pierce, Rockford, IL, USA), for the production of site-directed Abs. Two outbred New Zealand White rabbits received a primary immunization by an intramuscular injection of 400 μg of purified (102-121)-KLH in Titer Max Gold (CytRx Corporation, Atlanta, GA, USA). After 30 days, each animal received another intramuscular boost with 250 μg of rhGM-CSF in Titer Max Gold. After 3 days each rabbit was bled and the serum was collected as indicated above. Two sera anti-(102-121)-KLH were then obtained, labelled BB and BM.

Antibody screening

Enzyme-linked immunosorbent assay (ELISA). ELISA was carried out in two different ways. Ab-binding was assessed using the antigen immobilized on plastic plates (direct ELISA). Alternatively, binding was evaluated by using different antigens to inhibit the Ab binding to the immobilized antigen (competition ELISA).

For direct ELISA, plastic wells of a polyvinylchlorine (PVC) 96-well microtiter plate (Falcon 3911; Becton-Dickinson, Oxnard, CA) were coated with 0.2 μg /50 μl /well of rhGM-CSF or peptide-carrier protein conjugates, or 1.0 μg/50 μl/well of free peptide, in sodium carbonate buffer, pH 9.6, as described (52). The plates were incubated for 5 h at room temperature, and then saturated with 150 μl/well of 5% dry milk or 2% bovine gelatine in phosphate buffered saline containing 0.05%

Tween 20 (T-PBS), pH 7.4, for 2 h at room temperature. Varying concentrations of anti-rhGM-CSF or anti-peptide Abs, diluted in T-PBS, were then added to the wells (50 µl/well). After incubation for 3 h at room temperature or overnight at 4°C, plates were incubated with alkaline phosphatase(^{AP})-conjugated goat anti-rabbit IgG (^{AP}Goat anti-Rb IgG, Sigma), 50 µl/well, 1:1,000 in T-PBS, for 2 h at room temperature. Then 4-nitrophenylphosphate (PNPP, Merck AG, Darmstadt, Germany) 4 mg/ml in diethanolamine buffer, pH 9.8, was added as substrate. The optical density at 405 nm was measured with an automatic vertical beam reader (EASY-EIA Programmable MPT Reader, Giò De Vita, Milan, Italy). Plates were washed three times with T-PBS between each incubation step.

For competition ELISA, plastic wells of the microtiter plate were coated as indicated above, and saturated overnight at 4°C. At the same time the antisera to be tested, at a fixed dilution (capable of approximately 70-80% of the maximum binding to antigen coated wells), were mixed and incubated overnight at 4°C in separate tubes with varying concentrations of the inhibitor; 50 µl /well of these solutions (previously centrifuged at 2,000 RPM for 20 min) were added to the wells and incubated for 3 h at room temperature or overnight at 4°C. Plates were incubated with ^{AP}Goat-anti-rabbit IgG and PNPP was added as substrate, as indicated above. The optical density at 405 nm was then measured.

Western blot. Gel electrophoresis was run in 12% acrylamide in denaturating conditions, using sodium dodecyl sulfate (SDS). Samples of the protein of at least 1µg (10-20 µl) were boiled before electrophoresis was run for 5 min in a sample buffer containing SDS and β-mercaptoethanol. Proteins were transferred onto a nitrocellulose membrane (Biotrace NT 0.45 mm, Pall Gelman Sciences) by electroblotting. Membranes were saturated with 5% dry milk or 2% bovine gelatine in PBS, pH 7.4, for 2 h at room temperature, and then incubated with a solution of anti-rhGM-CSF or

anti-peptideAbs, diluted in PBS, for 3 h at room temperature or overnight at 4°C. Membranes were finally incubated with Goat anti-rabbit IgG, 1:1,000 in PBS, for 2 h at room temperature, and then with a solution of BCIP (5-bromine-4-chlorine-indolephosphate) and NBT (Nitro Blue Tetrazolium) as substrate, in sodium carbonate buffer, pH 9.6. Membranes were washed three times with PBS between each incubation step.

Bio-assays

The neutralizing Ab activity in the pre-immune and hyperimmune sera from rabbits 813, 814, BB and BM was tested in clonogenic assays performed with $1x10^5$ unfractionated human bone marrow mononuclear cells (BM-MNC) in 1 ml semisolid agar culture in Dulbecco's modified Eagle's medium (DMEM, Euroclone, West York, UK) containing 20% fetal bovine serum (FBS, Euroclone, West York, UK) and 0.3% agar. Colonies (> 20 cells) induced by adding rhGM-CSF (20 ng/ml/plate) with saline or the test serum were scored using an inverted Leitz microscope after 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air. Results are given as number of granulocyte-macrophage colonies (CFU-C) per plate in triplicates (-S.E.).

Molecular modeling

The hydrophobic profile was estimated as the running average (n=9) of the Kyte-Dolittle hydrophobicity index by the ExPASy web server (*www.expasy.org*). Visualization of 3-dimensional structures was done with VMD (53). Representations of the molecular surfaces were obtained with the program SURFER (54) integrated to VMD, using a 1.4Å probe. Accessible surface area for each aminoacid was estimated with the program NACCESS (55). Molecular dynamic simulations were used to predict the stability of the synthetic peptides. Crystalline structure of hGM-CSF was

used to define the initial atomic positions of synthetic peptides (Figure 1B). Mutations were introduced by the Tleap module of the Amber 7 (56). Peptide models were hydrated in an explicit water sphere of 27Å using Tleap. Molecular dynamic calculations were executed by the program NAMD (57), using AMBER-99 force field parameters. Models were extensively minimized by 20,000 steps, and energy change at the end of minimization procedure was less than 0.2 Kcal/mol/step in all peptide models. For molecular dynamics, an 10 Kcal/mol/Å harmonic constraint was applied to a 3Å shell of the water sphere, and all other atoms were left free. Models were gradually heated to 300°K, and free molecular dynamics were followed for 2 ns, and the structure was saved every 1 ps. Molecular dynamic trajectories were analyzed by the Ptraj module of Amber 7. The backbone of the structure of snapshots of the trajectories were fitted to the corresponding region of the crystallographic structure of hGM-CSF, and the root mean square deviation (rmsd) was estimated as a criteria of stability.

RESULTS

The C-terminal domain of hGM-CSF

The hydropaticity profile of the hGM-CSF is characterized by highly hydrophilic regions (low hydrophobicity index), near α-helices A and D (Figure 2A), which may be involved with the interaction of the polypeptide with specific neutralizing Abs of hyperimmune sera (26). Here, we concentrated on the region around the α-helix D, characterized by a hydrophilic N-terminus and a hydrophobic C-terminus (Figure 2B). The stabilization of an α-helix in the framework of the tertiary structure of the protein usually depends on the differential distribution of the polar and non-polar residues on the α-helix surface. However, a detailed characterization of the single residue's contribution to the immuno-reactivity cannot be deduced from the traditional average hydropaticity index profile. We expect that the residues directly involved with the Ab binding must be exposed to the external surface. Therefore, we estimated the accessible surface area for each aminoacid in this region. As shown in the upper panel of Figure 2B, the accessibility analysis of hGM-CSF crystallographic structure shows that, in α-helix D, residues E104, E108, K111, D112, L115 and V116 are the most exposed to the solution (>80Å^2). The importance of E21 of the α-helix A for interaction with the β-chain of the receptor was demonstrated (27). Similarly, E108 and D112, both hydrophilic and exposed to the solution, have been proposed as essential for interaction with the α−chain of the hGM-CSF receptor (28). Three residues of the loop after the α-helix D of hGM-CSF, F119, E123 and P124, are exposed on the surface. The vicinity of these residues to the exposed region of the α-helix D may suggest the participation of this region in the interaction with other molecules.

To analyze the role of these residues in C-terminus antigenicity, we synthesized peptides with partially overlapping sequences, analogous to the regions 93-110, 102-120 and 110-127 of the hGM-CSF, respectively (Figure 2B). We expected the isolated synthetic peptides to maintain the same conformation in solution as in the complete GM-CSF protein. A criterion for hypothesizing the structural stability of the synthetic peptides was based on the trajectory of the peptides by molecular dynamics simulations with explicit solvent. All peptides analyzed conserved the same conformation as the corresponding segment of the native hGM-CSF (Figures 3). The average of the configuration obtained in 2 ns molecular dynamic simulations was compared with the native crystalline structure of hGM-CSF. The root mean square deviation (rmsd) with respect to the native protein is shown in the table. The small rmsd (<2.5Å) estimated for all peptides considered herein may confirm that a significant destabilization on the conformation of the isolated peptides does not occur. This concept is confirmed by the NMR data obtained for the (13-31)-Gly-Pro-Gly-(103-116), indicating a highly conserved conformation, with respect to the hGM-CSF native polypeptide (51).

Antibody detection in sera

Sera obtained from rabbits before and after immunization were analyzed for the capacity of neutralizing the rhGM-CSF clonogenic activity on BM-MNC in soft agar assays as well as for the contents of Abs capable of specifically binding rhGM-CSF in Western blotting assays of Abs directed to bacterial toxins of other Escherichia Coli-related products which may contaminate rhGM-CSF preparations (20).

Bio-assays

Sera from the four pre-immunized rabbits, tested at varying dilutions from 1:200 to 1:4,000, did not inhibit the response of BM-MNC to added rhGM-CSF (142±12 CFU-C/1x10^5 BM-MNC/20 ng rhGM-CSF/14-days incubation). In contrast, hyperimmune sera from all four rabbits contained Abs blocking specifically CFU-C formation in a dose-dependent manner. At 1:2,000 dilution, all four hyperimmune sera completely inhibited granulocyte-macrophage colony formation from BM-MNC in soft agar assays (data not shown).

Anti-rhGM-CSF polyclonal antiserum

Western blot. Both antisera 813 and 814 from rabbits immunized against rhGM-CSF contained Abs able to strongly bind the rhGM-CSF. Through electrophoresis in denaturant conditions and successive transfer on a nitrocellulose membrane, it was shown that both sera contained Abs able to specifically bind the rhGM-CSF molecule (Figure 4).

ELISA. Peptide (102-121) was tested in direct and competition ELISA tests using sera of both rabbits 813 and 814 hyperimmunized against the rhGM-CSF. For this purpose the peptide (102-121) was adsorbed on microtitration plates as free peptide or conjugated to BSA (Figure 5A). Only one of the two sera (labeled 813) showed an appreciable specific affinity for the synthetic peptide (102-121) in direct ELISA. Instead, both sera contained Abs able to bind the synthetic peptide (14-24), analogous of the hGM-CSF N-terminal region, confirming our previous results (26). Of these two sera, neither contained Abs specific for the peptide (55-65), analogous to the region including the second α-helix of the hGM-CSF. These results showed that the peptide (102-121) had the same

three-dimensional structure as the corresponding sequence 102-120 on the native molecule, confirming the results obtained by molecular dynamics.

In direct ELISA tests with serum 813, the peptide [E108A](102-121) showed the same binding of the peptide (102-121) (data not shown). The antibody titer of this serum for the peptide [E108A](102-121) appeared comparable with the titer for the peptide (102-121). Affinity of the serum for the region 102-120 was not therefore modified by the substitution of the glutamate in position 108.

Rabbit 813 polyclonal serum bound the peptide (13-31)-Gly-Pro-Gly-(103-116) with an affinity comparable to peptide (14-24) (Figure 5B). Serum 814 showed an affinity for the peptides (13-31)-Gly-Pro-Gly-(103-116) and (14-24) comparable with serum 813 (data not shown). A possible explanation for the identical antibody titer for the peptides (13-31)-Gly-Pro-Gly-(103-116) and (14-24) is that the antibodies bind an antigenic region common to both the peptides.

Polyclonal serum 813 contained antibodies capable of binding the peptide [C121A](110-127) but not the peptide [C96A](93-110) (Figure 5B). Polyclonal serum 814, which in previous experiments did not contain antibodies able to bind (102-121), was shown in these experiments to contain Abs that bound the peptide [C121A](110-127), but not the peptide [C96A](93-110) (data not shown).

Competition ELISA. In competition tests using serum 813, the hGM-CSF and the peptide (102-121) were able to inhibit the binding of the polyclonal Abs to the (102-121) conjugated to BSA and immobilized on the microtitration plate. Inhibition was greater for the peptide conjugated to BSA than for the free peptide, because every BSA molecule was conjugated with approximately twenty molecules of (102-121). These results may be due to more stable immune complexes between antibodies and (102-121)-BSA, rather than between antibodies and free peptides (52).

rhGM-CSF was able to inhibit the binding of polyclonal Abs to the immobilized peptide better than the peptide (102-121). Therefore, anti-rhGM-CSF Abs recognized an epitope common to hGM-CSF and the (102-121), but they probably had a greater affinity for the conformation of the whole protein. These results also demonstrated, at least for one serum against hGM-CSF, that the region 102-120 is immunogenic. This characteristic may be conferred on the region by polar residues within its sequence.

In competition ELISA with serum 813, the peptide [E108A](102-121) showed an inhibition efficiency comparable to the peptide (102-121) (data not shown).

The peptide (13-31)-Gly-Pro-Gly-(103-116) did not inhibit the binding of the antibodies with the peptide (102-121) conjugated to BSA and immobilized on the plastic (data not shown). Therefore, the serum probably bound a region of the peptide (13-31)-Gly-Pro-Gly-(103-116) outside of the sequence (103-116), which includes the fourth α-helix.

Also, the peptides [C96A](93-110) and [C121A](110-127) were tested in competition ELISA, showing that serum 813 had an identical cross-reaction with the peptides (102-121) and [C121A](110-127) (data not shown). Instead, with serum 814, [C121A](110-127) inhibited the binding of Abs to the immobilized antigen less than (102-121) (data not shown). These results were obtained by immobilizing both (102-121)-BSA or [C121A](110-127). In competition tests, antibodies of both antisera as well did not bind [C96A](93-110) (Figure 5C). These results suggest that the C-terminal immunogenic region of hGM-CSF includes those amino acids following at least the residue in position 110.

Anti-(102-121)-KLH polyclonal antisera

Western blot. Through electrophoresis in denaturant conditions and successive transfer onto a nitrocellulose membrane, it was possible to show that antibodies of both these sera bound (102-121)-BSA but not Cys-BSA and other peptides conjugated to BSA (data not shown). Therefore, the two anti-peptide sera demonstrated a high specificity for peptide (102-121). Cross-reactivity of sera anti-(102-121)-KLH with the recombinant hGM-CSF was tested in Western blot: the antibodies specifically bound the rhGM-CSF molecule (Figure 4).

ELISA. Sera from both rabbits (BB, BM) immunized against (102-121)-KLH bound the peptide (102-121). One of the two sera (BB) showed a cross-reaction with a control peptide conjugated to albumin, by the linker maleimmide used for the conjugation of peptides with carriers, KLH or BSA. Therefore, a fraction of the Abs may be directed against the linker.

Both the polyclonal antisera anti-(102-121)-KLH were analyzed in direct ELISA with the whole recombinant protein. Both sera contained Abs able to bind an epitope on the surface of rhGM-CSF, suggesting that the epitope bound by these sera has the same structure of the analogous region on hGM-CSF and confirming the data obtained with sera anti-rhGM-CSF (data not shown).

In direct ELISA the sera anti-(102-121)-KLH bound the peptide [E108A](102-121) as well as the peptide (102-121) (data not shown). Therefore, the epitope bound by anti-peptide sera was not modified by the amino acid substitution.

Rabbit sera containing Abs directed against the peptide (102-121) were then tested with peptide (13-31)-Gly-Pro-Gly-(103-116). Serum BM Abs did not bind this peptide, and serum BB did not contain Abs able to appreciably bind the peptide, either (Figure 6A). Therefore, region (103-116) on the peptide (13-31)-Gly-Pro-Gly-(103-116) was not bound by anti-(102-121)-KLH Abs, showing that the immunogenic region of the peptide (102-121) comprises only its C-terminus.

Both sera showed a high Ab titer for the peptide [C121A](110-127) but negligible cross-reactivity with [C96A](93-110) (Figure 6A). These results indicate an epitope common to (102-121) and [C121A](110-127).

Competition ELISA. In competition ELISA with sera anti-(102-121)-KLH, the peptide (102-121) inhibited the binding of the Abs to (102-121) immobilized on the microtitration plate (data not shown).Region (102-121) was also able to inhibit the binding of these site-directed antibodies to adsorbed rhGM-CSF (Figure 6B).

In competition ELISA, recombinant hGM-CSF inhibited the binding of these site-directed antibodies with the adsorbed hGM-CSF (Figure 6B), but not with the peptide (102-121) immobilized on the plate (data not shown), suggesting that anti-peptide sera contained immunoglobulins specific for a linear epitope which was not easily accessible on whole hGM-CSF. Another possible explanation of these results could be the greater affinity of these Abs for the peptide rather than for the whole protein.

In competition ELISA with anti-peptide sera, [E108A](102-121) showed the same inhibition efficiency as (102-121), showing that Glu^{108} substitution is not important for epitope recognition and the presence of a hydrophobic amino acid does not influence the epitope binding (data not shown).

Then anti-(102-121)-KLH Abs and the peptides [C96A](93-110) and [C121A](110-127) were tested in competition ELISA (data not shown). In a preliminary experiment, with rhGM-CSF immobilized on the plate, only the peptide [C121A](110-127) effectively inhibited the binding of the Abs with the protein, whereas the peptide [C96A](93-110) did not inhibit binding. In the next experiment as well, with (102-121)-BSA adsorbed, only the peptide [C121A](110-127) inhibited binding.

DISCUSSION

The first and fourth α-helix, near the N-terminus and C-terminus respectively, are critical for interaction of the hGM-CSF with the α-chain of its receptor (28). Although the N-terminus and C-terminus are distant in the amino acid sequence, protein folding brings them close together (29).

Previous results showed immunogenicity of the synthetic peptide (14-24), analogous to the region 14-24 of the hGM-CSF N-terminus (26). Anti-rhGM-CSF antisera produced in our laboratory did not contain antibodies against the region comprising the second of the four α-helices of hGM-CSF. Therefore, we concluded that the second α-helix of hGM-CSF is not immunogenic. Consequently, the destruction of the α-helix conformation in the analogous region of the murine GM-CSF (with the insertion of a proline), does not inhibit the biological activity of recombinant protein (58). Other experiments suggested that a region near the fourth α-helix has a partial reactivity with monoclonal antibodies produced against the whole recombinant hGM-CSF molecule (26;52;59).

Understanding the hGM-CSF mechanism of action not only depends on the study of its effects on target cells, but also on its structure and its physical and chemical properties. In this way the studies were carried out by other researchers on the N-terminal regions of erythropoietin (60), TNF-α (61) and IFN-γ (62), and then on IL-2 (63) and GM-CSF (20;25;26;59).

For this study four rabbits were immunized: two against the rhGM-CSF; two against the peptide (102-121), analogous to region 102-120 of this cytokine, covalently conjugated to KLH. Hyperimmune sera from all four rabbits labeled 813, 814, BB and BM contained Abs able to neutralize specifically rhGM-CSF activity in the classical CFU-C forming assay. Sera of both rabbits 813 and 814 immunized against the whole protein contained Abs that specifically

recognized hGM-CSF, binding the epitope (14-24) but not (55-65). However, only one of these sera (rabbit 813) contained Abs that appreciably bound the peptide (102-121).

Sera of both rabbits BB and BM immunized against the peptide (102-121) contained specific Abs directed against (102-121) and recognized the whole rhGM-CSF molecule. Substitution of glutamate 108 with an alanine in the peptide (102-121) did not influence its immunogenicity, suggesting that this acidic residue is not essential for the binding of sera anti-rhGM-CSF and anti-(102-121)-KLH with hyperimmune polyclonal Abs.

In the peptide (13-31)-Gly-Pro-Gly-(103-116) two regions, (13-31) and (103-116), analogous to the first and fourth α-helix of the human cytokine respectively, were joined by the spacer (Gly-Pro-Gly) which allowed the two helices to assume an antiparallel conformation similar to the cytokine. Antibodies anti-(102-121) did not recognize the peptide (13-31)-Gly-Pro-Gly-(103-116). A possible explanation may be that the residues bound by antibodies on the region (103-116) are hidden by region (13-31) due to the folding of this synthetic peptide, but instead these antibodies cross-reacted with the whole rhGM-CSF molecule, where the regions (13-31) and (103-116) are adjacent as in (13-31)-Gly-Pro-Gly-(103-116). An alternative explanation is that the epitope of the α-helix D is located (or requires the presence) of the region beyond the residue 116. Antibodies anti-rhGM-CSF showed an appreciable and similar cross-reactivity with both peptides (13-31)-Gly-Pro-Gly-(103-116) and (14-24), but in competition ELISA the peptide (13-31)-Gly-Pro-Gly-(103-116) did not inhibit the binding of anti-protein antibodies to the immobilized peptide (102-121). On the contrary, rhGM-CSF inhibited this binding. Therefore, most probably the epitope recognized by antibodies anti-(102-121) and anti-rhGM-CSF does not include the entire region 103-116.

The antigenic determinant could therefore include part of the carboxylic terminus of the α-helix D, which was recognized by the antibodies directed against the whole hGM-CSF molecule. In order to

better characterize the immunogenic region of C-terminus, the peptides [C96A](93-110) and [C121A](110-127), analogous of regions 93-110 and 110-127 of the human cytokine respectively, were tested in both direct and competition ELISA. Results showed that the first of these peptides was not recognized by polyclonal antibodies directed against the region 102-120 and against the whole hGM-CSF. The peptide [C121A](110-127) was instead recognized by anti-peptide and anti-protein antibodies, with approximately the same affinity showed for the peptide (102-121).

Results described above show that antibodies anti-(102-121) recognized an epitopic domain which does not include the whole fourth α-helix D, but includes part of its C-terminal end. In fact, antibodies anti-(102-121)-KLH were not able to recognize the region (103-116) on the synthetic peptide (13-31)-Gly-Pro-Gly-(103-116).

Results then show that the neutralizing polyclonal anti-rhGM-CSF Abs recognize a region common to peptides (102-121) and [C121A](110-127), probably external to the sequence 103-116. Therefore, the C-terminal immunogenic region of hGM-CSF includes also a few residues successive to amino acid 116. In fact, even if in direct ELISA serum anti-rhGM-CSF was shown to contain Abs reacting against the three-dimensional peptide (13-31)-Gly-Pro-Gly-(103-116), subsequently, it was verified in competition ELISA that the epitope recognized by these Abs on this peptide was probably limited to the region 13-31.

Serum 814 polyclonal anti-rhGM-CSFAbs showed a greater affinity for the synthetic peptide [C121A](110-127) than for (102-121), according to the results obtained with serum 813. The C-terminus of rhGM-CSF includes five aminoacids next to residue 116, showing in this case a lower affinity for the region 116-120. In conclusion, the immunogenic portion of the hGM-CSF C-terminal region is represented by an epitope which only partially coincides with the peptide (102-

121), the critical immunogenic residues of the C-terminal region of hGM-CSF including part of the C-terminus of the α-helix D and five successive additional residues in the sequence 116-127.

Previous experiments demonstrated that the hGM-CSF C-terminal region 120-127 is not immunogenic. In fact, peptide (120-127), analogous to the region 120-127 of the hGM-CSF molecule, was not bound by blocking polyclonal Abs against rhGM-CSF (25). Considered together, these results showed that the immunogenic region of hGM-CSF C-terminus includes residues 116-120. The region 116-120 of hGM-CSF is apparently exposed on the surface of the protein (Figure 7). To reinforce this conclusion, previous studies identified an immunodominant epitope in proximity to the hGM-CSF N-terminal region (26), which is near the C-terminal region in the biologically active protein. Interestingly, some residues (F119, E123 and P124) of this region are particularly exposed on the surface of the molecule (Figure 7). Their vicinity to the exposed region of the α-helix D may also imply that this region is involved in the interaction with antibodies. Moreover, this domain plays an important functional role stimulating the production of Abs neutralizing the cytokine activity.

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FIGURE LEGENDS

Figure 1. A: Schematic representation of hGM-CSF. The four α-helices are shown as cylinders and the two β-sheets as arrows. Disulfide bridges are in yellow. The three-dimensional model is based on X-ray crystallographic data (29). **B:** Representation of surfaces of the three regions of hGM-CSF analyzed by competition with homologous synthetic peptides: 93-110, 102-121, 110-127. The color scale of the residues represents its surface accessibility (red for hidden residues, blue for the exposed ones).

Figure 2. A: Hydropaticity profile calculated along the whole hGM-CSF. An average of the hydrophobicity index for 9 amino acid around each residue is shown, together with a schematic representation of the four α-helices (boxes) and of the two β-sheets of the protein (thick lines). Synthetic peptide positions are indicated by discontinuous lines. **B:** Magnification of the hydropaticity profile for the region near to the C-terminus of hGM-CSF. A peak of high local hydrophobicity is present at the end of the fourth a-helix of the protein (103-116). The accessible surface for each residue is shown in the upper panel.

Figure 3. Topology of the α-helix-D, shown along the helical axis (**A**) and perpendicular to it (**B**). Color scale (blue-green-white) represents the accessibility of each residue. Observe that the less accessible residues (blue) are clustered on one face of the helix, while the more accessible residues (white) are in the opposite face. **C:** Superimposition of the molecular models of the synthetic peptides and the crystalline structure of the hGM-CSF molecule. Synthetic peptide models were obtained by averaging the snapshots obtained in a 2 ns molecular dynamics simulation. The crystalline hGM-CSF structure is drawn in white, peptide (102-121) in red, [E108A](102-121) in

blue, [C96A](93-110) in yellow and [C121A](110-127) in green. For all cases, rmsd < 2.5 Å. The table shows the root mean square deviation (rmsd) for the peptides with respect to the crystallographic structure of the hGM-CSF. Data are reported as the mean of the deviation of 2,000 conformations obtained during 2.0 ns molecular dynamic simulation, after an extensive minimization and equilibration to 300°K. Identical atoms are considered for calculations.

Figure 4. Western blotting analysis with rabbit sera anti-rhGM-CSF and anti-(102-121)-KLH, each tested at three dilutions. rhGM-CSF (4 μg/lane) was recognized by polyclonal antibodies of both the sera. BSA (4 μg/lane) was not bound.

Figure 5. ELISA with rabbit serum 813 anti-rhGM-CSF. **A:** Direct ELISA with rhGM-CSF, BSA and peptides adsorbed on wells (rhGM-CSF, BSA and conjugated peptides 0.2 μg per well; free peptides 1 μg per well). Polyclonal Abs bound the synthetic peptide (102-121), free or conjugated to BSA, and the peptide (14-24), but not the peptide (55-65) and a control peptide conjugated to BSA. **B:** Direct ELISA with free peptides adsorbed on wells (1 μg per well). Polyclonal Abs bound the peptides (102-121) and [C121A](110-127) , but not the peptide [C96A](93-110). The peptide (13-31)-Gly-Pro-Gly-(103-116) was recognized in a way comparable to the (14-24). **C:** Competition ELISA with rabbit serum diluted 1/1,000, corresponding to 75% of maximum O.D.. The conjugated peptide (102-121)-BSA was adsorbed on wells (0.2 μg per well). The free peptides (102-121) and [C121A](110-127) competed for the binding with polyclonal antibodies, whereas the free peptide [C96A](93-110) did not compete.

Figure 6. ELISA with rabbit serum BB anti-(102-121)-KLH. **A:** Direct ELISA with free peptides adsorbed on wells (1 μg per well). Site-directed polyclonal antibodies bound both the peptides (102-121) and [C121A](110-127), but not efficiently the peptides [C96A](93-110) and (13-31)-Gly-Pro-Gly-(103-116). **B:** Competition ELISA with rabbit serum diluted 1/1,000, corresponding

to 70% of maximum O.D.. rhGM-CSF was adsorbed on wells (0.2 μg per well). The peptide (102-121) and rhGM-CSF competed for the binding with polyclonal antibodies in a similar way.

Figure 7. Surface representation of the residues 93-127 of hGM-CSF. Color scale (blue-white-red) corresponds to the accessibility of residues. More accessible residues are indicated in the figure.

Figure 1

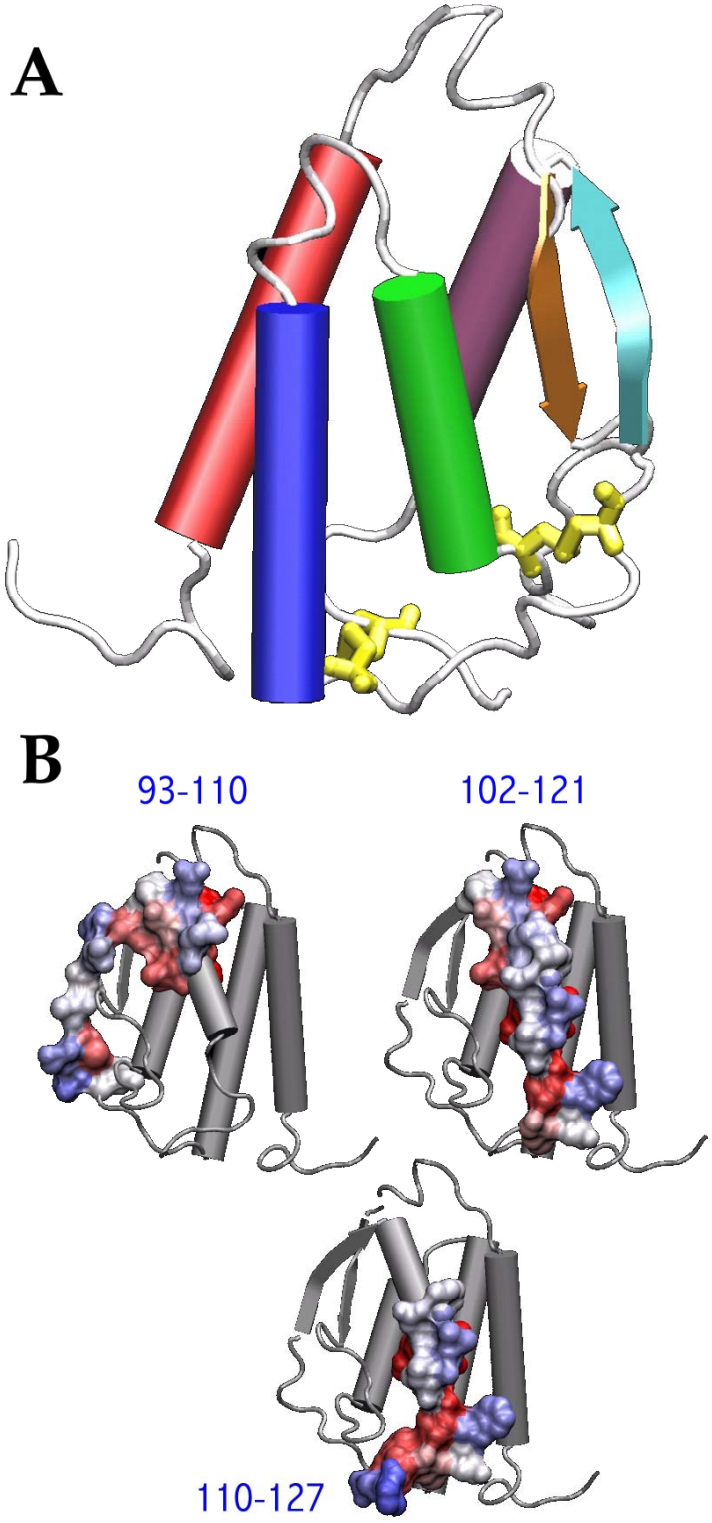


Figure 2

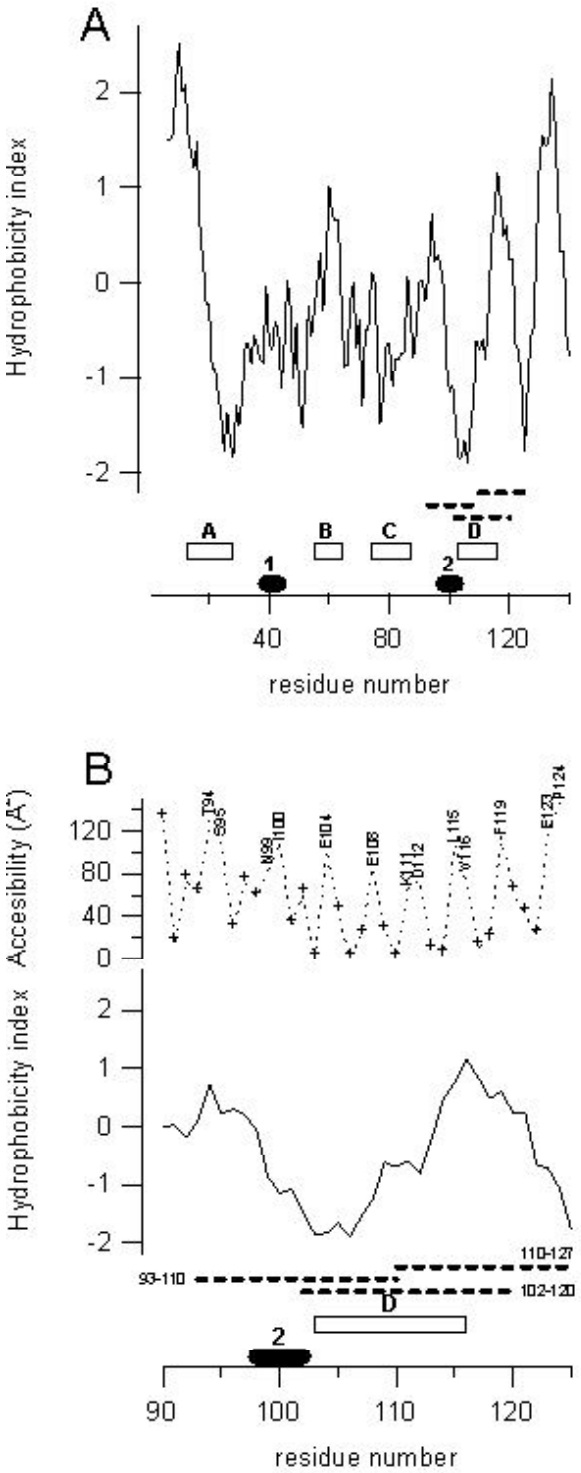
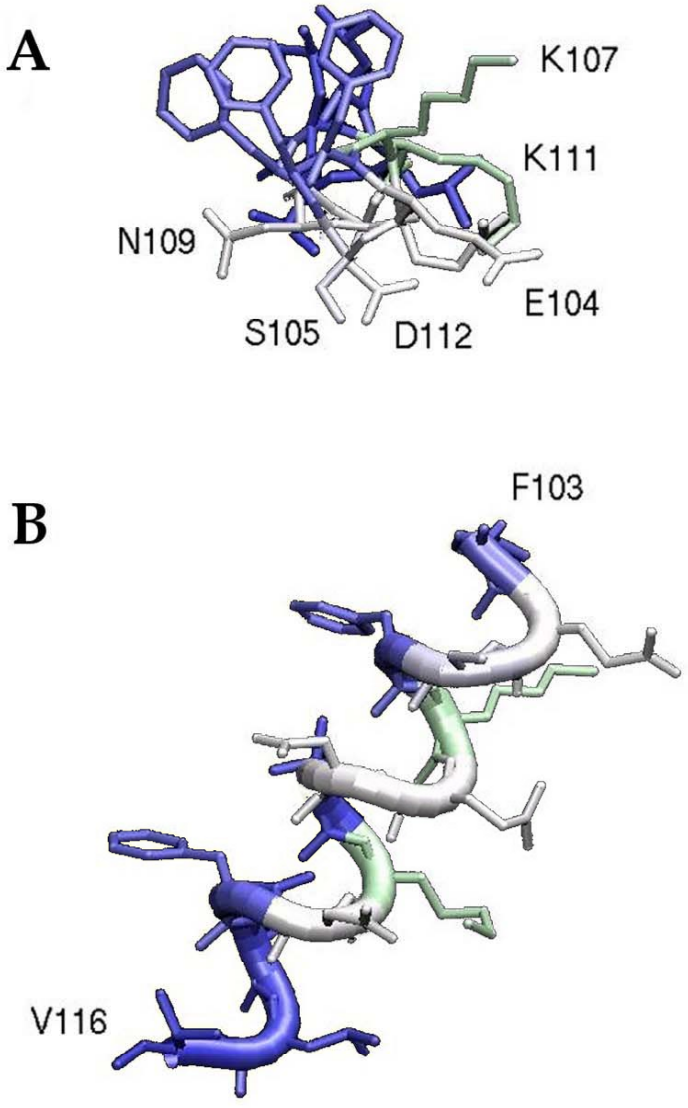
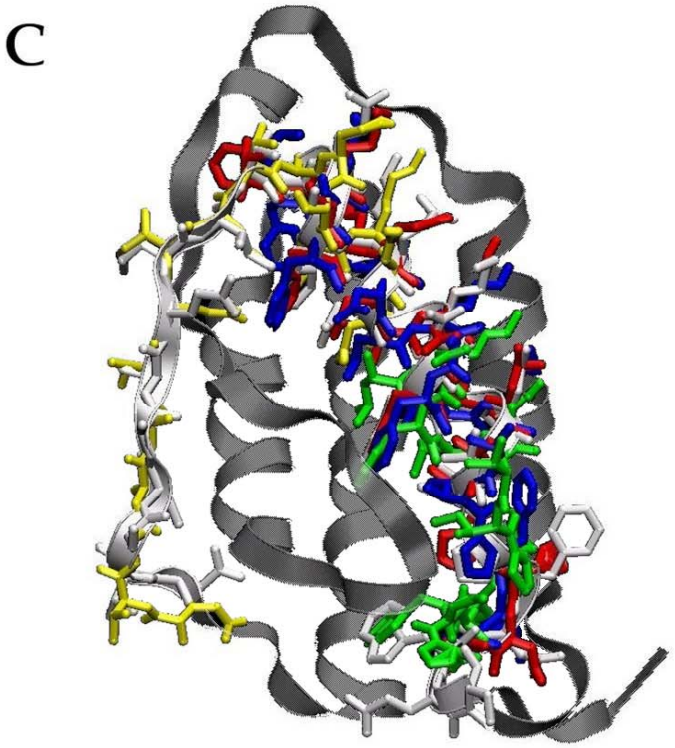


Figure 3





Peptide	rmsd (Å)
(102-121)	0.896
[E108A](102-121)	1.134
[C96A](93-110)	1.237
[C121A](110-127)	2.321
(13-31)-Gly-Pro-Gly-(103-106)	2.459

Figure 4

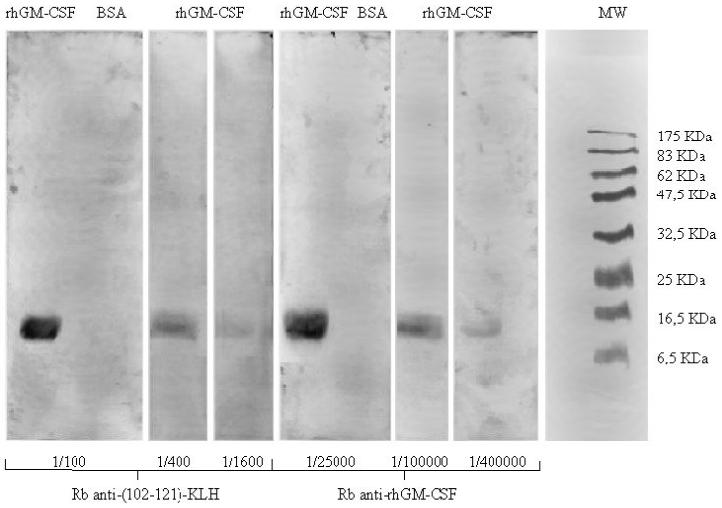


Figure 5

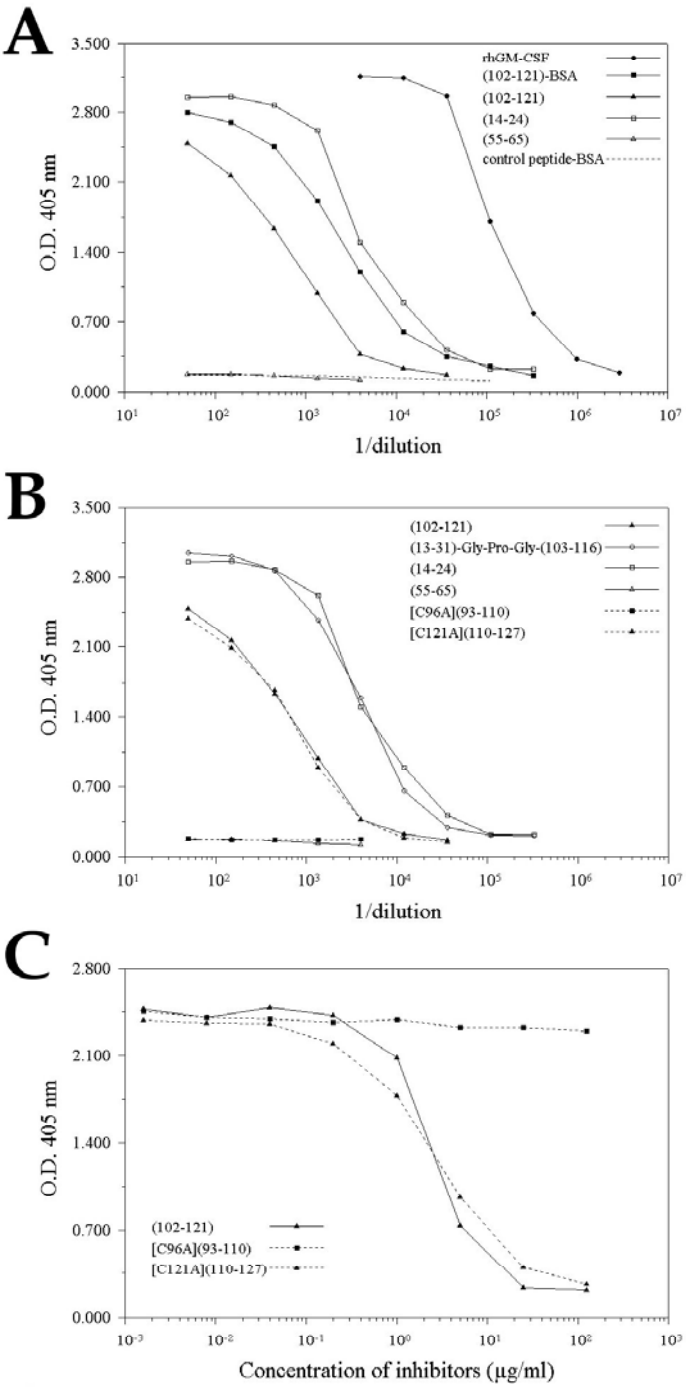


Figure 6

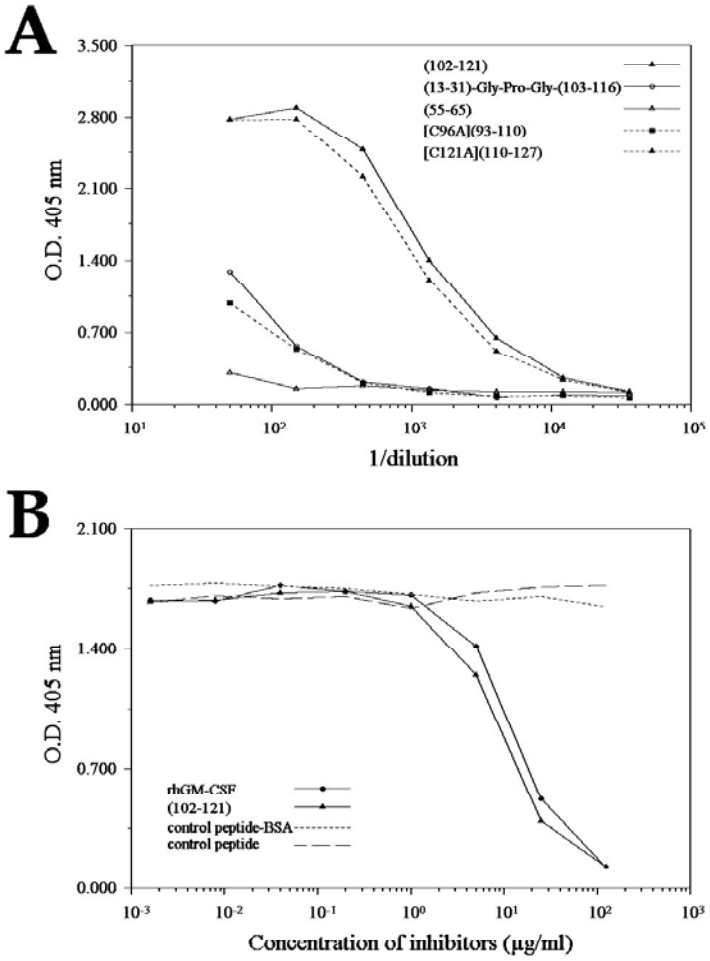
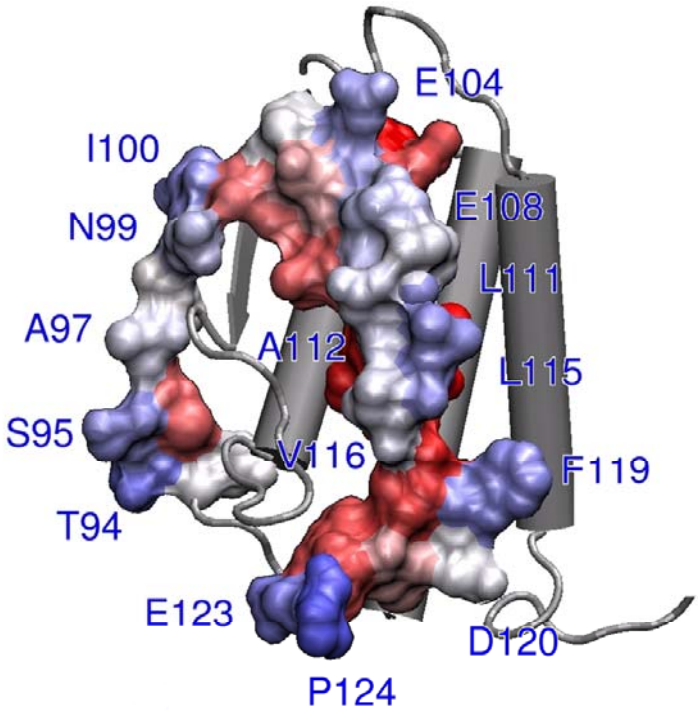


Figure 7



Identification of an antigenic domain near the C-terminus of human granulocyte-macrophage colony-stimulating factor and its spatial localization

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