The biological activity of granulocyte-macrophage colony-stimulating factor (GM-CSF) is modulated by the sulfated glycosaminoglycans (GAGs) heparan sulfate and heparin. However, the molecular mechanisms involved in such interactions are still not completely understood. We have proposed previously that helix C, one of the four α-helices of human GM-CSF (hGM-CSF), contains a GAG-binding site in which positively charged residues are spatially positioned for interaction with the sulfate moieties of the GAGs (Wettreich, A., Sebollela, A., Carvalho, M. A., Azevedo, S. P., Borojevic, R., Ferreira, S. T., and Coelho-Sampaio, T. (1999) J. Biol. Chem. 274, 31468–31475). Protonation of two histidine residues (His83 and His87) in helix C of hGM-CSF appears to act as a pH-dependent molecular switch to control the interaction with GAGs. Based on these findings, we have now generated a triple mutant form of human GM-CSF (mGM-CSF) in which three noncharged residues in helix C of the murine factor (Tyr83, Gln85, and Tyr87) were replaced by the corresponding basic residues present in hGM-CSF (His83, Lys85, and His87). Binding assays on heparin-Sepharose showed that, at acidic pH, the triple mutant mGM-CSF binds to immobilized heparin with significantly higher affinity than wild type (WT) mGM-CSF and that neither protein binds to the column at neutral pH. The fact that even WT mGM-CSF binds to heparin at acidic pH indicates the existence of a distinct, lower affinity heparin-binding site in the protein. Chemical modification of the single histidine residue (His15) located in helix A of WT mGM-CSF with diethyl pyrocarbonate totally abolished binding to immobilized heparin. Moreover, replacement of His15 for an alanine residue significantly reduced the affinity of mGM-CSF for heparin at pH 5.0 and completely blocked heparin binding to a synthetic peptide corresponding to helix A of GM-CSF. These results indicate a major role of histidine residues in the regulation of the binding of GM-CSF to GAGs, supporting the notion that an acidic microenvironment is required for GM-CSF-dependent regulation of target cells. In addition, our results provide insight into the molecular basis of the strict species specificity of the biological activity of GM-CSF. 

Granulocyte-macrophage colony-stimulating factor (GM-CSF), a 14-kDa monomeric glycoprotein that stimulates proliferation and differentiation of myeloid precursors into several cell types, such as monocytes/macrophages, granulocytes (1), and osteoblasts (2). The three-dimensional structure of the human form of this cytokine (3) shows that its main structural motif is a four-helix bundle, which forms a compact, globular fold. Although the structure of human GM-CSF (hGM-CSF) was determined more than 10 years ago, the precise molecular interactions involved in binding to its cellular receptor and the mechanism of signal transduction activated by such binding remain to be fully elucidated.

A high affinity protein receptor for GM-CSF has been described (4, 5), and its mechanism of activation has been characterized (reviewed in Ref. 6). However, several lines of evidence indicate that this is not the only receptor involved in the cellular responses to GM-CSF. Sulfated glycosaminoglycans (GAGs) have been implicated in the co-activation of growth factor receptors upon ligand binding (reviewed in Ref. 7). Moreover, the participation of cell-surface proteoglycans, mainly through their GAG moieties, in GM-CSF signaling and mitogenic activity has been reported by several groups (2, 8–10). In particular, heparan sulfate, a GAG mainly present on cell surfaces (11), and heparin, a structurally related soluble GAG, regulate the biological functions of GM-CSF. The interaction between GM-CSF and GAGs may also involve the GM-CSF high affinity protein receptor via formation of a complex containing one or more heparan sulfate chains. A similar type of ternary complex is known to be formed between fibroblast growth factor (FGF), a well known heparin-binding growth factor, its protein receptor, and GAGs (12, 13).

Although the role of proteoglycans in the modulation of GM-CSF signaling is clear, the precise binding site(s) for heparin/heparan sulfate in the GM-CSF molecule are still unknown. This may be partly related to uncertainties in the determination of the domains of GM-CSF involved in binding to its protein receptor (14). Moreover, despite the fact that conserved amino acid sequences corresponding to heparin-binding motifs have been identified (namely the XBBXXB and XBBXXBX motifs).
motifs, where B corresponds to a basic residue) (15), a large number of reports have also shown that heparin/heparan sulfate-binding sites in proteins are not always or necessarily defined by a unique sequence or structural motif (16). We have shown previously (17), by using a cell-free assay, that the interaction between hGM-CSF and heparin/heparan sulfate in solution leads to the formation of high molecular weight complexes. Most interestingly, the interaction requires an acidic pH, which led us to propose the existence of a pH-dependent GAG-binding site in hGM-CSF. That putative binding site is located in helix C of hGM-CSF and contains two histidine residues (His⁸⁶ and His⁸⁷) that become protonated at acidic pH and may mediate the interaction with negatively charged sulfate groups in GAGs (17). Based on those previous results, we have now constructed a “humanized” triple mutant form of murine GM-CSF (mGM-CSF) in which three noncharged residues in helix C (Tyr⁸³, Gln⁹², and Tyr⁹³) were replaced by the corresponding basic residues present in hGM-CSF (His⁸³, Lys⁹², and His⁹³), and we analyzed its interaction with immobilized heparin at different pH values. Taken together, the results presented here indicate a prominent role of helix C of hGM-CSF in the regulation of high affinity heparin binding, and we show that the widely conserved histidine residue (His¹⁵) present in helix A of GM-CSF also participates in the interaction with GAGs. Possible implications of our results in terms of the species specificity of the biological actions of GM-CSF are discussed.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptides—**Peptides (19-mers) corresponding to the amino acid sequences of wild type (WT) and mutated helix C of both murine and human GM-CSF (Table I) were purchased from Peptron (Daejeon, South Korea), and peptides corresponding to WT and mutated helix A of murine GM-CSF (Table I) were from Genemed Synthesis (San Francisco, CA). Their purities were ascertained by mass spectrometry. Circular dichroism analysis of the peptides was performed on a Jasco J-715 spectropolarimeter, as described below. Peptides were diluted in 20 mM K₂HPO₄, pH 7.5, to a final concentration of 0.1 mg/ml in the absence or in the presence of different concentrations of TFE (see “Results”).

Binding of the peptides to immobilized heparin was evaluated using a 1-m1 heparin-Sepharose 4B column (Amersham Biosciences) connected to an LC-10-AD high pressure liquid chromatography system (Shimadzu Scientific Instruments, Columbia, MD). Peptide solutions (0.1 mg/ml) were prepared in running buffer (20 mM acetate, pH 5.0, 0.5 M NaCl, pH 8.5) containing 20% (v/v) TFE, and 20 μl of each sample were loaded onto the column at a flow rate of 0.4 ml/min. Elution of the peptides was performed using a linear NaCl gradient, and the corresponding buffer and connected to the high pressure liquid chromatography system. The concentrations of purified WT and mutant mGM-CSF were determined using the BCA method (Pierce). The surface potential of all mGM-CSF constructs were checked by DNA sequencing using an Applied Biosystems 3730xl DNA Analyzer.

**Binding of WT and Mutant mGM-CSF to Immobilized Heparin—**Proteins (4 μM final concentration) were diluted in 20 mM sodium acetate, pH 5.0, or 20 mM potassium phosphate, pH 7.5, and were loaded onto a heparin-Sepharose 4B column (Amersham Biosciences). Column-bound fusion proteins were digested overnight at 4 °C with 10 IU of factor Xa (Amersham Biosciences). Column-bound fusion proteins were digested overnight at 4 °C with 10 IU of factor Xa (Amersham Biosciences), collected, and stored at −20 °C. The concentrations of purified GM-CSF samples were determined using the BCA method (Pierce).

**Structural and Functional Characterization of WT and Mutant mGM-CSF—**The purity and expression of recombinant hGM-CSF preparations were determined by 17% acrylamide SDS-PAGE and silver staining. Samples were also analyzed by Western blotting using an anti-mGM-CSF polyclonal antibody (Sigma) and the ECL detection kit (Amersham Biosciences). Commercial mGM-CSF (Peprotech, Rocky Hill, NJ) was used as a positive control. Circular dichroism measurements were performed at 23 °C on a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD), kindly made available by Dr. J. L. Silva, Federal University of Rio de Janeiro) using 0.1-cm path length quartz cells. Protein samples were diluted in 20 mM K₂HPO₄, pH 7.0, to the final concentrations indicated in the legends to the figures. The CDPro package (lamar.colostate.edu/~sreeram/CDPro/main.html) was used to calculate secondary structure contents from CD data. Intrinsic fluorescence emission spectra of WT and mutant mGM-CSF (10 μM) diluted in 20 mM K₂HPO₄, pH 7.0, were measured at 23 °C on an ISS (ISS Inc., Champaign, IL) PC-1 spectrofluorometer, in linear mode.

**Chemical Modification with DEPC—**WT mGM-CSF (4 μM) was diluted in 20 mM potassium phosphate, pH 7.0, and was incubated for 5 min at 23 °C with 50 μM of freshly dissolved DEPC (Sigma). Formation of carbamylated histidine was confirmed by measuring the increase in absorbance at 242 nm (20). To verify the extent of chemical modification, samples were purified using ZipTips (Millipore, Billerica, MA) immediately after modification and were analyzed by MALDI-MS using a Voyager-DE PRO spectrometer (Applied Biosystems, Foster City, CA) in linear mode.

**Electrodes for Surface Potential of hGM-CSF—**The surface potential was calculated using the Adaptive Poisson-Boltzmann Solver software package (21) and images were generated using PyMol 0.97 (22). The atomic coordinates for hGM-CSF were obtained from the Protein Data Bank entry 1CGS. Calculations were performed assigning either a neutral or a positive charge to His residues (see text). A probe sphere of 1.4 Å radius was used to generate the solvent-accessible surface. The dielectric constants used for protein and solvent were 20 and 80, respectively, and the system temperature was maintained at 310 K.
RESULTS

We have shown previously that the interaction of hGM-CSF with various GAGs, including heparan sulfate and heparin, leads to the formation of high molecular weight complexes revealed by light-scattering measurements (17). In that study, we also proposed the existence of a GAG-binding site in helix C of hGM-CSF. On the other hand, inspection of the amino acid sequence of mGM-CSF reveals that three basic residues that are thought to be important for heparin binding to helix C of hGM-CSF (His83, Lys85, and His87) are replaced by noncharged residues (Tyr83, Gln85, and Tyr87) in the murine protein. This is in line with the observation that formation of high order molecular complexes between human GM-CSF and heparin is much more robust than that observed with murine GM-CSF (17).

Interaction of Peptides Corresponding to Helix C of GM-CSF with Heparin—In order to gain insight into the possible roles of His83, Lys85, and His87 in the interaction of helix C of hGM-CSF with heparin, we initially used synthetic peptides corresponding to the 19-amino acid sequence of helix C in hGM-CSF or a “triple mutant” peptide in which those three basic residues were replaced by alanine residues (Table I). CD analysis of the secondary structures of the synthetic peptides showed that both WT and mutant peptides assume helical conformations in aqueous solution in the presence of increasing concentrations of TFE (data not shown). This indicates that the amino acid replacements introduced in the mutant peptide did not alter the helix propensity of helix C of hGM-CSF. In order to maintain a helical structure of the peptides, we routinely added 20% (v/v) TFE to the samples prior to heparin binding assays.

The relative affinities of the synthetic peptides for heparin were estimated from the concentrations of NaCl required to elute the peptides from a column containing immobilized heparin (Fig. 1A). The assays were carried out at both acidic pH (pH 5) and at pH 8.5, well above the pK value of histidine, to ensure that the two His residues present in the wild type peptide would be in a nonprotonated state. Reflecting the lack of the three basic residues that were replaced by alanines, the mutant human peptide presented a lower affinity for heparin than the WT peptide; at pH 5.0, NaCl concentrations of ~675 and 725 mM, respectively, were required to elute the mutant and WT peptides from the column. Most interestingly, the affinity of the WT peptide for heparin was substantially decreased at pH 8.5, and became very similar to the affinity of the mutant peptide. These results indicate that protonation of His residues of the WT peptide at acidic pH plays an important role in the modulation of heparin binding affinity. Furthermore, consistent with our previous proposal (17), the results obtained with the triple mutant peptide also show that Lys72 and Lys74, located at the N terminus of helix C of GM-CSF, also contribute to heparin binding.

We next investigated the interaction of synthetic peptides corresponding to the amino acid sequence of helix C of murine GM-CSF with immobilized heparin. In addition to the WT peptide, we used a triple mutant peptide in which three non-charged residues were replaced by the corresponding basic residues found in hGM-CSF (Table I). CD analysis showed that both WT and triple mutant murine peptides assumed helical conformations in the presence of TFE (data not shown). As described above, 20% (v/v) TFE was routinely used in heparin binding assays. At pH 5.0, the peptide corresponding to WT helix C of mGM-CSF bound to the heparin column with an affinity similar to that observed for the peptide corresponding to the mutated human helix C (Fig. 1). Most interestingly, at pH 5.0, the triple mutant murine helix C peptide bound to heparin with an affinity comparable with that found for the WT human helix C peptide (Fig. 1). These results clearly indicate the importance of His83, Lys85, and His87 in modulating high affinity heparin binding of the peptides. Furthermore, consistent with the requirement of His protonation for interaction with heparin, the affinity of the mutant murine peptide for immobilized heparin was markedly reduced at pH 8.5 (Fig. 1B).

Structural Characterization and Interaction of WT and Triple Mutant mGM-CSF with Heparin—Based on the results obtained with the synthetic peptides, we created a humanized mutant form of mGM-CSF in which three noncharged residues in helix C of the murine factor were replaced by the corresponding basic residues present in hGM-CSF (Y83H, Q85K, and Y87H). Recombinant WT and triple mutant mGM-CSF were expressed in E. coli and were purified from inclusion bodies using 6 x urea followed by progressive dialysis, as described previously for the purification of recombinant murine (23) and human GM-CSF (24). The purities and identities of both WT and mutant mGM-CSF were confirmed by SDS-PAGE and Western blot analysis (Fig. 2).

The α-helix content of mGM-CSF is estimated to be ~47% (25). CD measurements of our recombinant proteins (Fig. 3A) revealed helical contents of 43 and 46% for WT and triple mutant mGM-CSF, respectively, in excellent agreement with
the value reported previously. In addition, the similarity in helical contents found for WT and mutant mGM-CSF indicates that the three amino acid replacements did not alter the native structure of mGM-CSF. Intrinsic fluorescence measurements and size-exclusion chromatography analysis of the recombinant proteins showed that both WT and triple mutant mGM-CSF presented fluorescence emission spectra with intensity maxima at ~350 nm (Fig. 3B) and eluted as monomeric globular proteins in the calibrated column (not shown). These results are also in agreement with previous reports for mGM-CSF (26).

Recombinant WT and triple mutant mGM-CSFs were also assayed for their abilities to induce proliferation of murine myeloid cells. In this assay, we used the FDC-P1 cell line, which is strictly dependent upon GM-CSF or interleukin-3 (27). WT and triple mutant mGM-CSF induced proliferation of FDC-P1 cells to similar extents as a commercial preparation of mGM-CSF used as a positive control (Fig. 3C). It should be noted, however, that the FDC-P1 cell line grows in suspension in the culture medium, and, different from normal myeloid cells, its proliferation induced by mGM-CSF is not strictly dependent on interaction with extracellular matrix elements or with molecules present on the surface of co-cultivated cells. This makes it difficult to compare directly the regulation of GM-CSF responses of FDC-P1 and normal myeloid cells, and may also explain why our mutant GM-CSF induced proliferation of the cells to the same extent as WT mGM-CSF.

By having established the correct folding and biological activities of recombinant WT and triple mutant mGM-CSF, we investigated the interaction of these proteins with immobilized heparin. This approach has been widely used for the determination of the affinities of several proteins, including growth factors, for heparin (28–30). NaCl concentrations of 730 and 880 mM, respectively, were required to elute WT and triple mutant mGM-CSF from heparin-Sepharose at pH 5.0 (Fig. 4, upper panel). This shows that the introduction of the three basic residues in helix C significantly increased the affinity of the mutant form of mGM-CSF for heparin. Somewhat surprisingly, however, even WT mGM-CSF, which lacks the three basic residues in helix C, interacted with immobilized heparin with quite high affinity at acidic pH. On the other hand, when the column was equilibrated at pH 7.5, both WT and triple mutant mGM-CSF eluted in the flow-through of the column, indicating that these proteins do not bind to heparin at neutral pH (Fig. 4, lower panel). Taken together, these data suggest that the interaction between WT mGM-CSF and heparin is pH-dependent, as demonstrated previously for hGM-CSF (17), and possibly regulated by histidine ionization in the acidic to neutral pH range.

**Chemical Modification of WT GM-CSF with DEPC—WT murine GM-CSF contains a single histidine residue located at position 15 in helix A of the protein (3). To investigate further the role of His15 in the interaction of mGM-CSF with heparin, we carried out chemical modification of this amino acid residue using DEPC. This reagent reacts quite specifically with histidine residues in proteins, producing an adduct containing an N-carbethoxylation in the imidazole ring that blocks protonation (20, 31). This modification generates a 72-Da mass increment in the protein and can also be followed by the increase in absorbance at 242 nm because of the carboxethoxyhistidine adduct. Treatment of WT mGM-CSF (4 μM) for up to 30 min with DEPC at concentrations ranging from 10 to 100 μM did not cause any changes in absorbance at 242 nm. However, incubation of WT mGM-CSF with 500 μM DEPC for 5 min at 23°C caused an increase of ~0.01 in absorbance at 242 nm (data not shown). Based on the reported value of ε$^{242}$ for carboxethoxyhistidine (3,200 μM$^{-1}$ cm$^{-1}$ (20)), we estimated that the extent of modification of His15 approached 80% under these conditions. We also checked the derivatization of mGM-CSF by using mass spectrometry. DEPC treatment induced the formation of a major mono-carbethoxylated species presenting a mass increment of ~72 Da relative to the native protein (Fig. 5). A minor peak showing a mass increment of ~144 Da, corresponding to a di-carbethoxylated species, was also present. Secondary, less specific carbethoxylation reactions of DEPC with other amino acid side chains have been described for other DEPC-modified proteins (20, 31). It should be noted, however, that although mass spectrometry provides accurate measurements of the molecular mass of the species formed upon DEPC treatment, it most likely does not provide a precise quantitative estimate of the actual ratio between unmodified and carboxethoxylated GM-CSF. Because of the carboxethoxylation, it is reasonable to expect

![Fig. 2. Purification of recombinant WT and triple mutant murine GM-CSF.](image)

A, silver-stained 17% SDS-PAGE showing single bands with the expected molecular weight in samples of purified WT and triple mutant mGM-CSF. The M$_r$ markers (M) used are cytochrome c (12 kDa) and ovalbumin (45 kDa). B, Western blot analysis of purified proteins using anti-mGM-CSF antibody. Commercial mGM-CSF was used as a positive control. The same amount of protein was loaded in the three lanes.

![Fig. 3. Structural and functional characterization of recombinant WT and triple mutant mGM-CSF.](image)

A, far-UV circular dichroism (A) and intrinsic fluorescence emission spectra (B) of WT (solid line) or triple mutant (dashed line) mGM-CSF. In both cases, protein concentration was 10 μM. Measurements were performed as described under “Experimental Procedures.” C, mitogenic activity of WT and triple mutant mGM-CSF (10 ng/ml) on FDC-P1 cells. Commercial mGM-CSF (10 ng/ml) was used as a positive control. Proliferation was measured using the 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) assay. Results correspond to means ± S.D. of three independent experiments. In all conditions (commercial, WT, or mutant mGM-CSF), proliferation was significantly higher ($p < 0.001$) than the control containing no growth factor.
that the relative gas phase proton affinity of the nitrogen atom of the imidazole ring of His15 modified in mGM-CSF is significantly decreased, which may result in differences in ionization energy between the unmodified and modified proteins (32). Such differences could be reflected in the intensities of the peaks, thus impeding precise quantification of peak intensities using MALDI-MS.

Interaction of Chemically Modified mGM-CSF with Heparin—To evaluate the effect of His15 DEPC modification on heparin binding, we submitted the modified WT mGM-CSF sample to affinity chromatography in heparin-Sepharose at pH 5.0. Remarkably, DEPC-treated WT mGM-CSF did not bind to immobilized heparin, eluting in the flow-through of the column (Fig. 6, dotted line), whereas a control sample not treated with DEPC eluted with ~700 mM NaCl (Fig. 6, solid line).

Interaction of Peptides Corresponding to Helix A of GM-CSF with Heparin—The results presented above indicated a significant role of His15 in the heparin binding activity of GM-CSF and led us to evaluate the binding of peptides corresponding to the sequence of helix A of murine GM-CSF (see Table I for amino acid sequences) to heparin. Peptides corresponding to both the WT sequence of helix A and a mutant in which His15 was replaced by an alanine residue were synthesized. Both peptides were analyzed by CD in order to assess their secondary structure contents. As in the case of the synthetic peptides corresponding to helix C, helix A 19-mer peptides demonstrated a high propensity to form α-helical structures in the presence of 20% (v/v) TFE (data not shown).

Most interestingly, the mutant His15 → Ala helix A peptide did not bind to immobilized heparin at pH 5.0 and eluted in the flow-through of the column (Fig. 7). On the other hand, the peptide corresponding to the WT sequence of murine helix A presented a more complex behavior regarding its heparin binding activity at pH 5.0. Some of the material interacted with quite high affinity to immobilized heparin, eluting at ~760 mM NaCl (Fig. 7). However, an additional peak eluting at ~600 mM NaCl was also present (Fig. 7). The presence of the two peaks in the elution profile of WT murine helix A peptide may be related to peptide aggregation during the chromatographic run. Indeed, we have observed that this peptide tends to aggregate in aqueous solution in the absence of TFE (data not shown), suggesting that it might undergo partial aggregation upon dilution of TFE during the chromatographic analysis.

Structural Characterization and Interaction of (H15A)mGM-CSF with Heparin—In order to investigate directly the role of His15 in the binding of murine GM-CSF to heparin, we have constructed a single mutant form of mGM-CSF containing the H15A substitution, and we examined its ability to interact with immobilized heparin. This construct was expressed and purified in E. coli exactly as done for WT and triple mutant mGM-CSF. CD analysis of the purified protein revealed a 47% con-
Fig. 6. Modification with DEPC blocks heparin binding to WT mGM-CSF. WT mGM-CSF (4 μM) was treated with DEPC as described in the legend to Fig. 5. The modified protein (dotted line) was then diluted in 20 mM sodium acetate, pH 5.0, and was immediately loaded onto the heparin-Sepharose column equilibrated at pH 5.0. Control, unmodified WT mGM-CSF (solid line) was submitted to the same treatments as the modified sample in the absence of DEPC. Runs and elution were carried out as described in the legend to Fig. 4.

FIG. 7. Interaction of peptides corresponding to helix A of mGM-CSF with immobilized heparin at pH 5.0. The peptides used correspond to WT (solid line) or H15A mutant (dotted line) murine helix A sequence. The column was equilibrated with 20 μM sodium acetate, pH 5.0, and samples were prepared in the same buffer containing 20% (v/v) TFE. Peptides were eluted by a linear NaCl gradient ranging from 0.5 to 1 M in 10 column volumes (indicated by the dashed line).

DISCUSSION

Considerable evidence implicates the sulfated GAGs heparan sulfate and heparin in the modulation of the biological activity of GM-CSF (9, 10, 33, 34). However, to date no GAG-binding sites in GM-CSF have been structurally identified. We have proposed previously the existence of a heparin-binding site located in helix C of human GM-CSF (17). We have now shown that protonation of two histidine residues (His83 and His87) in helix C of hGM-CSF plays a significant role in the regulation of heparin binding. In addition, we found that a conserved His residue (His15), located in helix A of GM-CSF, is also involved in the interaction with heparin.

A similar role of histidine protonation has been described in the binding of murine tryptase to heparin in secretory granules of mast cells (29). That protein binds with high affinity to immobilized heparin at pH 5.0 but not at neutral pH. More generally, histidine ionization has also been implicated in the control of the interaction of proteins with molecules other than GAGs, such as in virally induced membrane fusion at the acidic environment of the endosomal compartment (35).

Comparison of the amino acid sequences of GM-CSF from various sources reveals a high degree of homology. In particular, murine and human GM-CSF share 54% identity in amino acid residues. This percentage is even higher if conservative amino acid substitutions are considered. Most interestingly, however, murine GM-CSF specifically lacks His86 and His87, which appear to modulate the affinity of helix C of hGM-CSF for heparin (Fig. 1A). Those two residues, in addition to another basic amino acid (Lys85), are replaced by noncharged residues in helix C of mGM-CSF. Additional evidence for the role of those His residues in the binding of hGM-CSF to heparin was provided by light-scattering measurements, which indicated that murine GM-CSF does not interact with heparin at acidic pH as efficiently as human GM-CSF (17). Direct indication that His83 and His87 modulate heparin binding to hGM-CSF was obtained by site-directed mutagenesis of murine GM-CSF. Introduction of those two residues plus Lys85 in helix C of mGM-CSF was sufficient to increase the heparin-binding affinities of the protein (Fig. 4A) as well as of a synthetic peptide corresponding to the amino acid sequence of helix C (Fig. 1B).

Most of the heparin/heparan sulfate-binding sites that have been described in proteins contain the basic residues lysine and arginine, which participate in electrostatic interactions with negatively charged sulfate or carboxylate groups in GAGs (36, 37). Therefore, the contributions of Lys72, Lys74, and Lys85, all of which are located in helix C of both human and murine GM-CSF, to heparin binding must also be considered. Together with His83 and His87, those Lys residues define a heparin-binding motif in helix C of hGM-CSF following the criteria described by Margalit and co-workers (38). Residues Lys72 and Lys74 are also present in all the helix C peptides tested (Table I), and they are likely responsible for the interaction with heparin that is observed with both WT and mutant human and murine helix C peptides at both acidic and basic pH values (Fig. 1). However, the pH dependence of heparin binding to both human and murine helix C peptides (Fig. 1), as well as to WT and triple mutant mGM-CSF (Fig. 4), indicates that His residues play a prominent role in the modulation of the interaction between heparin and GM-CSF. Further evidence indicating that His protonation modulates GM-CSF/heparin interaction was provided by chemical modification of the single histidine residue (His15) in murine GM-CSF with DEPC, which abolished heparin binding at pH 5.0 (Fig. 6). Similar results have been reported for the interaction of murine tryptase (mMCP-7) with heparin (29). In that case, single mutations of three different His residues into Glu were sufficient to prevent the binding of mMCP-7 to heparin. Those three His residues were proposed to form a His-rich motif on the surface of the protein that becomes positively charged at acidic pH and triggers interaction with heparin (29). Most interestingly, in the three-dimensional structure of human GM-CSF (Protein Data Bank entry 1CSG), His15 is positioned in close proximity to the two His residues present in helix C, His86 and His87. Calculation of the surface electrostatic potential of hGM-CSF revealed that at neutral pH, when the His residues are expected to be essentially noncharged, most of its surface is markedly negative (Fig. 9, A and C). In particular, the face of the protein where the three His residues are exposed is preferentially neutral (Fig.
FIG. 8. Interaction of (H15A)mGM-CSF with immobilized heparin at pH 5.0. WT (solid line) and (H15A)mGM-CSF (dotted line) samples (4 μM) were prepared in 20 mM sodium acetate, pH 5.0, and were loaded onto the heparin-Sepharose column previously equilibrated with the corresponding buffer. The dashed line corresponds to a linear NaCl gradient ranging from 0 to 1 M. Inset, far-UV circular dichroism of purified (H15A)mGM-CSF diluted in 20 mM phosphate buffer, pH 7.0. Protein concentration was 2 μM.

FIG. 9. Surface electrostatic potential of human GM-CSF at neutral and acidic pH. Calculations were performed considering the His residues in both neutral (A and C) and protonated forms (B and D). A and C and B and D show two different orientations of the molecule rotated 180° about the vertical axis with respect to each other. Molecular surfaces are colored by electrostatic potential in units of kT following the color bar code shown below the figure. Red surfaces correspond to negative charges, and blue denotes positive charges. Histidine residues 15, 83, and 87 are labeled in yellow, cyan, and green, respectively, in A and C and in the insets. Insets show a detailed view of the area inside the transparent gray circle in both orientations of the molecule. The surfaces were generated using the Adaptive Poisson-Boltzmann Solver software package and visualized using PyMol. The atomic coordinates for hGM-CSF were from Protein Data Bank entry 1CSG.

TABLE II

| Species      | Amino Acid Sequence |
|--------------|---------------------|
| Human        | VDEVA-IQEARRLLN28   |
| Gibbon       | VDEVA-IQEARRLLN28   |
| Porcine      | VHVIDA-IQEALSLLN29  |
| Bovine       | VHVIDA-IQEALSLLN29  |
| Sheep        | VHVIDA-IQEALSLLN29  |
| Red deer     | VHVIDA-IQEALSLLN29  |
| Dog          | VHVIDA-IQEALSLLN29  |
| Cat          | VHVIDA-IQEALSLLN29  |
| Guinea pig   | VHIDTA-NLEALSLLN29  |
| Rat          | VVEA-IQEARRLLN28    |
| Mouse        | VVEA-IQEARRLLN28    |

results showing that His<sup>15</sup> is also important for modulation of the affinity of interaction of murine GM-CSF with heparin, we argue that this residue is generally involved in heparin-binding sites in GM-CSF from different species. In this regard, it is interesting to note that a recent work (39) has shown that surface-exposed histidine residues mediate the interaction of murine tryptase 6 with heparin and, consequently, its biological activity.

The precise mechanisms involved in the regulation of the biological activities of growth factors by GAGs are still under investigation. The main hypotheses have been reviewed by Gallagher (40) and include the following: (i) an increase in local concentration of growth factors through binding to abundant GAGs on the surface of target cells; (ii) conformational changes in the growth factor induced by interaction with GAGs, which might trigger high affinity receptor binding; and (iii) stabilization of the growth factor-receptor complex, such as found with PFG. We have shown previously that hGM-CSF undergoes a slight conformational change induced by the interaction with heparin at acidic pH (17). It is also possible that, in vivo, heparan sulfate proteoglycans stabilize the interaction between GM-CSF and its high affinity protein receptor through cross-bridging, and may even directly participate in the intracellular signaling induced upon GM-CSF binding, as detected in the interaction of GM-CSF and the proteoglycan syndecan-2 in osteoblasts (10). In that case, phosphorylation of Tyr residues located in the cytoplasmic region of the proteoglycan was found to be important for the
activation of a protein kinase implicated in GM-CSF-induced intracellular signaling.

An important question that arises from the present work concerns the requirement of an acidic environment for efficient interaction between GM-CSF and GAGs. In contrast with the case of tryptase, in which protein-GAG interaction takes place in an intracellular acidic compartment, the interaction between GM-CSF and GAGs occurs in the pericellular space, where both the GM-CSF receptor and heparan sulfate proteoglycans are found. We have proposed previously that the negatively charged extracellular microenvironment generated by the accumulation of molecules such as glycolipids, glycoproteins, and proteoglycans in close proximity to the cell membrane (41) would create a local pH gradient with the external face of the membrane being acidic (17). Recent observations have provided experimental support to this hypothesis; the contact points between GM-CSF-dependent murine FDC-P1 cells and the stromal cells that secrete GM-CSF concentrate negative charges from glycolipids containing sialic acid groups, suggesting that the microenvironment where GM-CSF and its high affinity receptor interact is indeed acidic (34). Moreover, ongoing studies show that the GM-CSF receptor co-localizes with gangliosides on the surface of hematopoietic precursor cells.2 Also of interest is that modulation of the activity of FGF on endothelial cells by highly sialylated glycolipids has been demonstrated (42). In addition, other physiological mechanisms also seem to be regulated by gangliosides in a similar manner. For example, the inhibition of nerve regeneration mediated by myelin-associated glycoprotein has been shown to involve clustering of gangliosides (43). In another model, the assembly of laminin on the surface of astrocytes in culture as well as the laminin-induced neuritogenesis of primary neurons cultured on them have been shown to be controlled by the local acidic pH provided by sialic acid-containing lipid rafts on the membranes of astrocytes (44, 45).

GM-CSF is one of the major growth factors controlling myelopoiesis (1), which occurs both in the bone marrow, under physiological conditions, and in peripheral tissues, in pathological situations (8). Myelopoiesis has been shown to be dependent on the molecular composition of the local intercellular environment, in particular on the structures of proteoglycans present (8, 33, 46). Here we have described a mechanism of regulation of the interaction of GM-CSF with GAGs based on histidine ionization. Taken together, these results support the notion that the molecular nature and the distribution of proteoglycans and gangliosides on the membranes of target cells are responsible for their selective response to GM-CSF stimulus, which is closely related to the control of myelopoiesis. Finally, despite the high degree of sequence homology between murine and human GM-CSF, their biological activities are highly species-specific (47), i.e. cells that are dependent on human GM-CSF are not activated by murine GM-CSF or vice versa. Based on the present findings, it seems possible that the differential binding to GAGs might play an important role in the species specificity of this growth factor. In this context, the widely conserved His15 residue may play a role in the modulation of GAG binding to a common, constitutive GAG-binding site in GM-CSF molecules from different species. On the other hand, the nonconserved His residues located in helix C may contribute to the regulation of GAG binding in a species-specific context of target cell interaction.

2 F. Guma and R. Borovec, personal communication.

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