Acidic pH Modulates the Interaction between Human Granulocyte-Macrophage Colony-stimulating Factor and Glycosaminoglycans*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) controls growth and differentiation of hematopoietic cells. Previous reports have indicated that the mitogenic activity of GM-CSF may be modulated by the glycosidic moiety of proteoglycans associated with the membrane of stromal cells. In this work, we have performed in vitro studies of the interaction between GM-CSF and glycosaminoglycans. The addition of heparin promoted a marked blue shift in the fluorescence emission spectrum of GM-CSF as well as a 30-fold increase in the intensity of light scattering, which indicates formation of large molecular weight complexes between the two molecules. Interestingly, heparin-induced changes in the spectral properties of GM-CSF were only observed at acidic pH. The dependence on acidic pH, together with a strict dependence on glycosaminoglycan sulfation and the fact that high ionic strength destabilized the interaction, indicates that the association between GM-CSF and glycosaminoglycans is mediated by electrostatic interactions. These interactions probably involve sulfate groups in the glycosaminoglycans and positively charged histidine residues in GM-CSF. We propose that negatively charged glycolipids present on the plasma membrane of the hematopoietic and/or the stromal cell could promote an acidic microenvironment capable of triggering interaction between GM-CSF and membrane-bound proteoglycans in vivo.

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† The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; GAG, glycosaminoglycan; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; TNS, 2-(p-toluidino)naphtalene-6-sulfonic acid.

In the present study, we have used spectroscopic techniques to investigate the interaction between recombinant human GM-CSF and GAGs in vitro. We have found that GM-CSF undergoes a conformational change upon acidification of the pH of the medium and that the acid-stable conformation is the one mediating in vitro binding with GAGs. In addition, we show that negatively charged phospholipid vesicles (at neutral pH) induce similar conformational changes in GM-CSF as those promoted by acid pH. Based on these observations, we propose that, under physiological conditions, the acidic environment necessary to trigger the effective interaction between GM-CSF and GAGs may be provided by negatively charged groups present on the external surface of the plasma membrane of the interacting cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human GM-CSF was a generous gift from Amgen Inc. (Thousand Oaks, CA). Porcine intestinal mucosa heparin (average molecular mass 15 kDa), bovine intestinal mucosa heparin

2 R. Borojevic, unpublished results.
Effect of Heparin on the Intrinsic Fluorescence of GM-CSF

Human GM-CSF possesses two tryptophan residues (23). Here, we have used intrinsic fluorescence measurements to investigate conformational changes of GM-CSF induced by interaction with GAGs. The fluorescence emission spectrum of GM-CSF presented a peak at 349 nm (corresponding to an average emission wavelength of 358.1 nm) (Fig. 1A). The addition of 1 μg/ml heparin did not promote any significant spectral shift.
and only a modest (30%) decrease in fluorescence intensity (Fig. 1A). Previous studies using FGF-1 and FGF-2 have shown that, although a decrease in FGF fluorescence was observed upon interaction with heparin at neutral pH (24), it was only at acidic pH that heparin induced a shift of the FGF fluorescence spectrum (25). In order to examine whether a similar behavior was exhibited by GM-CSF, we investigated the effect of heparin at pH 4. In this condition, heparin promoted a 6-nm blue shift of the emission spectrum (with a change in average emission wavelength from 355.0 to 349.0 nm) (Fig. 1B). It is noteworthy that pH acidification by itself caused a 3.1-nm blue shift relative to the emission spectrum measured at neutral pH, which indicates that GM-CSF undergoes a conformational change upon acidification.

**Interaction between GM-CSF and Heparin Monitored by Light Scattering**—Examination of the fluorescence spectra obtained at pH 4 showed that heparin promoted a marked increase in the intensity of light scattering, seen at the blue edge of the fluorescence emission (Fig. 1B, inset). This increase in light scattering indicates an increase in the size of the particles in solution (26). Interaction between one molecule of GM-CSF and one molecule of heparin (15 kDa) would not lead to the increase in light scattering observed in Fig. 1B (inset). The observed increase in light scattering probably reflects the formation of large complexes, involving simultaneous binding of several units of both GM-CSF and heparin molecules. In order to confirm the formation of high order complexes, we have centrifuged the samples at acidic pH and compared the protein contents in solution before and after centrifugation (Fig. 2). Centrifugation for 10 min at 16,000 × g was sufficient to sediment all of the protein content of the samples, as shown by both a dramatic decrease in light scattering (Fig. 2A) and the complete disappearance of the 14-kDa band in SDS-polyacrylamide gel electrophoresis of centrifuged samples (Fig. 2B). These results show that high molecular weight complexes are formed between GM-CSF and heparin at acidic pH.

Light scattering was measured at 400 nm following stepwise additions of heparin to GM-CSF (Fig. 3). At pH 7.5, the addition of heparin did not promote any increase in light scattering, whereas at pH 4.0 light scattering increased progressively, reaching a maximal enhancement of about 30-fold at 1 μg/ml heparin. The concentration of heparin necessary to promote 50% of maximal complex formation was ~300 ng/ml.

To determine the heparin/GM-CSF stoichiometry in the complex, we carried out the experiment shown in the inset of Fig. 3. FITC-labeled heparin (1 μg/ml) was added to a sample of GM-CSF at pH 4. The sample was centrifuged for 10 min at 16,000 × g, and the intensity of FITC fluorescence remaining in the supernatant was measured after neutralization.

Only 10% of the initial fluorescence was detected in the supernatant, which indicates that approximately 90% of the heparin molecules added were incorporated into the complex together with GM-CSF. These results indicate that the K_d for heparin binding to GM-CSF at acidic pH is approximately 300 ng/ml, or 20 nM (using the average molecular mass for heparin of 15 kDa).

**pH Dependence of the Interaction between GM-CSF and GAGs**—In order to determine the optimal pH for complex formation, we investigated changes in light scattering as a function of pH (Fig. 4). Complexes were formed by the addition of 1 μg/ml heparin to a GM-CSF sample at pH 4. This sample was subsequently neutralized by stepwise additions of Tris buffer (Fig. 4, closed circles). Neutralization promoted a progressive decrease in light scattering, indicating disruption of previously formed complexes. From these data, an apparent pH of 5.2 for complex formation was determined. An interesting finding was that when heparin was first added to GM-CSF at pH 7.4 with...
subsequent acidification of the medium, only a slight increase in light scattering was detected (Fig. 4, open circles). This result indicates that the two initial states (GM-CSF at pH 4 plus heparin and GM-CSF at pH 7.5 plus heparin) cannot be readily interconverted by changing the pH, i.e. that these two states are not in fast equilibrium (see “Discussion”).

Specificity of the Interaction between GM-CSF and GAGs—In order to investigate the contributions of size and degree of sulfation to the interaction of GAGs with GM-CSF, we have compared light scattering increases induced by 15-kDa heparin with those induced by 3-kDa heparin and by chemically (N + O)-desulfated 3.5-kDa heparin (Fig. 5A). Additions of 15- or 3-kDa heparin promoted maximal increases of light scattering of 30- and 22-fold, respectively. In both cases, maximal levels were obtained at 1 μg/ml heparin, with \(K_{0.5}\) values of approximately 300 and 400 ng/ml, respectively. The addition of 1 μg/ml of (N + O)-desulfated heparin hardly promoted any increase in light scattering, indicating that sulfation is strictly required for the interaction of heparin with GM-CSF. In line with these results was the observation that the addition of high concentrations of NaCl promoted complete dissolution of the GM-CSF-GAG complexes (IC\(50\) ~0.5 m; data not shown). This indicates that the association between GM-CSF and GAGs depends on electrostatic interactions, probably involving sulfate groups in GAGs and basic residues in GM-CSF. It is interesting to note that physiological concentrations of salt (150 mM NaCl) did not prevent formation of the GM-CSF-GAG complexes (not shown).

In order to determine whether interaction with GM-CSF was specific for heparin, we examined light scattering increases promoted by other GAGs (Fig. 5B). Heparan sulfate promoted a 42-fold increase in light scattering. The apparent affinities for heparan sulfate and 15-kDa heparin were approximately similar. Chondroitin and dermatan sulfate, although they also promoted significant increases in light scattering (22- and 13-fold, respectively), presented lower apparent affinities for GM-CSF than heparin or heparan sulfate (Fig. 5B). These data indicate that the interaction between GM-CSF and GAGs is not solely determined by the degree of sulfation but rather depends on other structural requirements. Nevertheless, the importance of sulfate groups was confirmed by the observations that hyaluronic acid, a nonsulfated GAG, did not cause any increase in light scattering, whereas dextran sulfate, a polysulfated non-GAG molecule, did (Fig. 5B).

TNS Binding to GM-CSF at Neutral or Acidic pH—The fact that detectable interaction between GM-CSF and GAGs occurred only at acidic pH (Fig. 4), along with the finding that acidification promoted a blue shift of the fluorescence emission of GM-CSF (Fig. 1), indicated that the growth factor presented different conformations depending on pH. To further investigate this point, we have compared the binding of TNS to GM-CSF at pH 4 and 7.5. The hydrophobic fluorescent probe TNS has been used to monitor conformational changes in proteins, since it is fluorescent only upon binding to hydrophobic sites and is essentially nonfluorescent when free in aqueous solution (27). As shown in Fig. 6, binding to GM-CSF, revealed by a significant increase in TNS fluorescence, was observed only at acidic pH. At neutral pH, no binding of TNS to GM-CSF was observed. This result confirms that GM-CSF undergoes pH-induced conformational changes, which result in exposure of TNS binding sites at acidic pH.

Binding of TNS to GM-CSF in the Presence of Acidic Phospholipid Vesicles—The fact that the interaction between GM-CSF and GAGs reported here was only observed at acidic pH could, in principle, argue against a possible physiological role for this phenomenon in hematopoiesis, since the pH of the extracellular matrix where these two molecules physiologically interact is neutral. On the other hand, it has been reported that the effective pH near the surface of vesicles containing acidic phospholipids is about 2 pH units lower than the pH of the bulk aqueous phase (28). In order to investigate whether an acidic membrane surface could promote the conformational changes in GM-CSF necessary for interaction with GAGs, we measured TNS binding to GM-CSF at pH 7.5 in the presence of vesicles containing neutral, acidic, or an equimolar mixture of neutral and acidic phospholipids (Fig. 7). Binding of TNS to GM-CSF was higher in the presence of acidic phosphatidylserine vesicles.

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**Fig. 4.** pH dependence of the interaction between GM-CSF and heparin. Light scattering was measured for GM-CSF alone (●, ■) or for GM-CSF in the presence of 1 μg/ml 15-kDa heparin (○, ○). Heparin was added to GM-CSF either at pH 4.0 (●) or at pH 7.5 (○). Progressive pH neutralization or acidification (see arrows) were obtained by stepwise additions of Tris or HCl, respectively, as described under “Experimental Procedures.”

**Fig. 5.** Glycosaminoglycan specificity for the interaction with GM-CSF. Light scattering of GM-CSF was measured at pH 4.0 upon the stepwise addition of different GAGs. A, 15-kDa heparin (●), 3-kDa heparin (○), or (N + O)-desulfated 15-kDa heparin (×). B, 15-kDa heparin (●), heparan sulfate (○), chondroitin sulfate (×), dermatan sulfate (■), hyaluronic acid (□), and dextran sulfate (△).
Interaction between GM-CSF and Glycosaminoglycans

A sample of GM-CSF was bound to a heparin-Sepharose column at pH 5. After extensive washing with acidic buffer, the pH was brought to 7.5, and GM-CSF was eluted (Fig. 8A). Noteworthy is the fact that GM-CSF loaded onto the column at neutral pH did not bind to the heparin matrix (Fig. 8B). When the sample collected from the column from panel A was tested on a clonogenic proliferation assay, similar cell growth was observed as compared with the growth induced by a nonacidi-fied sample of GM-CSF (Fig. 9). This demonstrates that the interaction of GM-CSF with heparin at acidic pH does not inactivate the mitogenic activity of the growth factor.

**DISCUSSION**

Heparin-like GAGs are known to stimulate the proliferative activity of GM-CSF (13, 15). In this work, we report that the interaction between GAGs and GM-CSF can be modulated by acidic pH. We propose that, in an acidic environment, GM-CSF undergoes a conformational transition that enables it to interact with GAGs, giving rise to a multimeric complex.

Acid pH-induced conformational changes in GM-CSF were revealed both by a blue shift in the intrinsic fluorescence emission and by increased binding of the hydrophobic probe TNS (Figs. 1 and 6). Intrinsic fluorescence has previously been used to monitor pH-induced conformational changes of other growth factors, such as G-CSF (30) and FGF-I (25). For FGF-I at neutral pH, the fluorescence is dominated by tyrosine emission, since the emission of the single tryptophan residue is strongly quenched. At acidic pH, quenching of tryptophan fluorescence in FGF is relieved, resulting in an increase in fluorescence. The addition of heparin promoted a further fluorescence increase, which was accompanied by a blue shift of the maximal emission (25). The intrinsic fluorescence of GM-CSF is dominated by tryptophan emission regardless of pH or of the presence of heparin (Fig. 1). Both pH acidification and heparin binding caused blue shifts of the fluorescence emission of GM-CSF (Fig. 1). Unlike changes in fluorescence intensity that may be related to quenching of tryptophan emission by interaction with neighboring amino acids, a spectral blue shift unambiguously reflects a decrease in the exposure of tryptophan residues to the aqueous medium. Conversely, a red shift would indicate an increase in solvent exposure, as generally observed upon protein denaturation (31). The fact that both heparin binding and pH acidification promoted blue shifts of the fluorescence emission indicated that formation of a complex between GM-CSF and GAGs does not result from denaturation of the factor. The fact that GM-CSF retained its biological activity after binding to a heparin-Sepharose gel at acidic pH (Fig. 9) further supports the view that the acid-induced complex formation reported here does not result from irreversible denaturation of the growth factor.

TNs is known to interact with hydrophobic regions in proteins (27). The finding that TNS binding to GM-CSF occurred only at acidic pH suggests that acidification leads to exposure of hydrophobic pockets in the protein, which were not exposed at neutral pH. In addition to its hydrophobic character, TNS also presents a sulfonate group, which could mediate electrostatic interactions with basic residues in the growth factor. The finding that TNS binding was abolished when GM-CSF was previously denaturated by urea (data not shown) indicates that, although a contribution from electrostatic interactions cannot be completely discarded, it is very likely that hydrophobic regions in GM-CSF are made available upon pH acidification and determine the interaction with TNS.

The fact that association between GM-CSF and GAGs was blocked by high ionic strength indicates that electrostatic interactions play a central role in stabilizing the complex. The negative charges that participate in electrostatic interactions...
could be either carboxylate or sulfate groups in GAGs (32). However, the lack of ability of chemically desulfated heparin and nonsulfated hyaluronic acid to mediate complex formation indicates that sulfate groups are probably the ones that contribute the negative charges to the interaction. On the other hand, positive charges are probably contributed by side chains of basic amino acids in GM-CSF. Since interaction occurs only at acidic pH and histidine is the only amino acid to switch from neutral to positive below pH 7, one could expect participation of histidine residues in the heparin-binding site(s) of GM-CSF. The pK_{a} of free histidine is approximately 6, which is close to the apparent pK_{a} of 5.2 determined upon neutralization of acid-induced GM-CSF-heparin complexes (Fig. 4). The small discrepancy between the two values probably reflects the well known fact that ionizable side chains can exhibit changes in their apparent pK_{a} depending on the physicochemical properties of the surrounding protein environment. Thus, it is likely that the histidine residues of GM-CSF that participate in the interaction with GAGs are located in environments favoring their protonation at lower pH values than histidine alone in solution.

Based on a comparison of heparin-binding sequences in a variety of proteins, Cardin and Weintraub (33) proposed that defined amino acid sequences, XBBBXXBX and XBBXXB (where B is a basic residue, either lysine or arginine, and X is any amino acid residue), were common to heparin-binding sites. Margalit and co-workers (34) later proposed an alternative heparin-binding motif, in which the critical requirement for heparin binding was the presence of two basic amino acids located 20 Å apart and facing opposite sides of either an α-helix or a β-sheet. More recently, Hileman and co-workers (35) proposed a new consensus sequence, where approximation of basic residues arising from turns of the protein backbone was also taken into account. We have investigated the presence of such heparin-binding sites in GM-CSF, noting that in this particular case (i.e. acidic pH) histidine residues should be considered basic. We have not found either Cardin and Weintraub (33) or Hileman et al. (35) sequences. However, we found that one of the α-helices of GM-CSF, helix C, contains one histidine residue (His^{83}) located approximately 20 Å apart from a lysine residue (Lys^{84}) on opposite sides of the helix (Fig. 10). In addition, another lysine residue (Lys^{85}) and another histidine residue (His^{86}) are present within the 20-Å distance range between the two flanking basic amino acids, which is a further characteristic of the motif described by Margalit and co-workers (34). Furthermore, helix C does not contain any acidic residue that could destabilize heparin binding and has an amphipathic nature (with polar and nonpolar residues facing opposite sides of the helix), as also proposed in the model of Margalit et al. (34). Based on these observations, we believe that helix C might contain the putative heparin-binding site that mediates the acidic-pH-dependent complex formation between GM-CSF and GAGs.

In the present study, we have not determined the precise molecular weight of the GM-CSF-heparin complexes. However, the observation that maximal complex formation occurred when a 1 μg/ml concentration of 15-kDa heparin was added to 5 μg/ml GM-CSF, together with the fact that 90% of the GAG molecules were incorporated into the complex (Fig. 3), allows us to calculate that the approximate stoichiometry in the complex is five molecules of GM-CSF per molecule of GAG (considering an average molecular mass of 15 kDa for heparin). A heparin molecule of 15 kDa contains approximately 50 monosaccharide units. Thus, according to the stoichiometry estimated above, one GM-CSF molecule would bind to a GAG region corresponding to about 10 monosaccharides if the complex contained only one GAG molecule. This result would be in good agreement with previous data reported for FGF. In the latter case, although only 6 monosaccharide units are necessary for binding, 8–10 units are required for regulation of the biological activity (10, 36–38). It should be pointed out, however, that a complex between one 15-kDa heparin molecule and five GM-CSF molecules would have a molecular weight of ~85,000, which is not compatible with the light scattering and sedimentation data reported here (Figs. 2–5). Our experimen-

**Fig. 8. GM-CSF binding to heparin-Sepharose.** Chromatograms obtained for GM-CSF samples that were either loaded at pH 5 and eluted at pH 7.5 (A) or directly loaded at pH 7.5 (B) are shown. In both cases, the column was loaded with 1-ml samples containing 10 μg/ml pure GM-CSF. Buffers were changed as indicated by the arrows.
tional data indicate that interaction at acidic pH leads to formation of high molecular weight complexes, probably involving various molecules of GAG simultaneously interacting with several GM-CSF molecules. This interpretation, however, would predict the existence of a second binding site for heparin in GM-CSF. We have identified only one heparin-binding motif in GM-CSF (Fig. 10), but it is possible that other heparin-binding sites, not matching the structural motifs described previously (33–35), may also be present in GM-CSF.

Alternatively, hematopoietic growth factors are known to form homodimers or to dimerize upon receptor binding (39, 40). It is possible that dimers of GM-CSF could bridge two heparin molecules, promoting formation of a network complex of high molecular weight. This could possibly explain the lack of reversibility (i.e. lack of fast equilibrium) between the neutralization and acidification curves shown in Fig. 4. It is possible that the difference between GM-CSF samples diluted at pH 4 (which are capable of forming high molecular weight complexes in the presence of heparin) and GM-CSF samples diluted at pH 7.5 (which do not form high molecular weight complexes in the presence of heparin, even upon subsequent acidification of the pH) could be related to the presence of GM-CSF dimers or higher order oligomers at acidic pH. Once dissociated by dilution to 5 μg/ml ( ~0.35 μM) in neutral buffer, these GM-CSF oligomers might not reassociate by pH acidification, either because of the addition of heparin or because the oligomerization process does not reach equilibrium in the time frame of the experiment. Phenomena related to the lack of fast equilibrium for oligomerization have been previously described for protein dimers and other oligomers (41, 42). It is interesting to note that incubation of the acidified sample (open circle far left in Fig. 4) for 48 h at room temperature did not lead to any increase in light scattering, further indicating the lack of (or very slow rate of) equilibrium between different types of complexes formed under different solution conditions.

Acidic pH has previously been shown to modulate interaction between amyloid peptides and GAGs (43, 44), which is considered to be of potential relevance in amyloidogenesis. In order to support a cellular role for an acidic-pH-triggered interaction between GAGs and GM-CSF in vitro, it would be necessary to identify a physiological correspondent of pH acidification, given that the pH of the extracellular environment is essentially neutral. We have considered the possibility that an acidic pH microenvironment could be found in the close vicinity of the outer face of cell membranes and caused by negative membrane lipids. This hypothesis was supported by our finding that at neutral solution pH in the presence of vesicles containing acidic phospholipids (phosphatidylserine), GM-CSF underwent conformational changes similar to those triggered by acidic pH in bulk aqueous solution (spectral blue shift of intrinsic fluorescence and increased TNS binding). In this study, we have used phosphatidylserine as a model of acidic lipids. However, it is known that phosphatidylserine tends to concentrate in the internal face of the membrane bilayer in normal cells (45). On the other hand, sialylated glycosphingolipids are known to form clusters on the outer leaflet of the plasma membrane (46, 47) and could be responsible for local pH acidification. In this regard, it is important to note that sialylated glycosphingolipids have been shown to modulate the interaction between growth factors such as FGF, epidermal growth factor, and platelet-derived growth factor and their receptors (48–51). Although the mechanism of such modulation is not fully understood, glycolipids have recently been hypothesized to affect low affinity heparan sulfate FGF binding sites (52).

Additional support for the hypothesis that an acidic microenvironment could trigger the effective interaction between GM-CSF and GAGs is given by the observation that cationic ferritin, a marker of negatively charged extracellular domains, accumulates at the site of contact between GM-CSF-dependent FDCP-1 cells and the liver connective tissue cells that secrete GM-CSF and support hematopoiesis in vitro.

In summary, we propose that clusters of negative lipids at the membranes of either the stromal or the hematopoietic cell (or both) could lead to a local decrease in pH and induce a conformational change in GM-CSF, which would then become capable of interacting with GAGs. This could lead to concentration of GM-CSF at the cell surface and more effective presentation to its protein receptor.

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FIG. 10. Proposed GAG-binding site in GM-CSF. Left panel, three-dimensional structure of GM-CSF, highlighting helix C in yellow. Right panel, representation of helix C in GM-CSF, with His and Lys residues colored in orange and red, respectively. The structures were generated using RasMol and the Protein Data Bank coordinates for human GM-CSF.
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