Heparin Binding Induces a Conformational Change in Pigment Epithelium-derived Factor*

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Pigment epithelium-derived factor (PEDF) is a noninhibitory serpin found in plasma and in the extracellular space. The protein is involved in different biological processes including cell differentiation and survival. In addition, it is a potent inhibitor of angiogenesis. The function is likely associated with binding to cell surface receptors in a heparin-dependent way (Alberdi, E. M., Weldon, J. E., and Becerra, S. P. (2003) BMC Biochem. 4, 1). We have investigated the structural basis for this observation and show that heparin induces a conformational change in the vicinity of Lys178. This structural change was evident both when binding to intact heparin and specific heparin-derived oligosaccharides at physiological conditions or simply when exposing PEDF to low ionic strength. Binding to other glycosaminoglycans, heparin-derived oligosaccharides smaller than hexadecasaccharides (dp16), or type I collagen did not affect the structure of PEDF. The conformational change is likely to expose the epitope involved in binding to the receptor and thus regulates the interactions with cell surface receptors.

EXPERIMENTAL PROCEDURES

Materials—Heparin, hyaluronic acid, chondroitin sulfate, heparinase, ovalbumin, porcine trypsin, heparin-BSA, and o-phenylenediamine dichloride were from Sigma. PEDF and type I collagen were purified as described previously (7, 16). Heparin-derived unsaturated tetrasaccharide (dp4) and octasaccharide (dp8) were from Neoparin, Inc. (Alameda, CA), and hexadecasaccharide, (dp16) was from Dextra Laboratories Ltd. (Reading, UK).

SDS-PAGE—Polyacrylamide gel electrophoresis was performed in 5–15% gradient gels using the glycine/2-amino-2-methyl-1,3-propanediol-HCl buffer system (17).

N-terminal Amino Acid Sequence Analysis—PEDF fragments were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) (18), and analyzed by automated Edman degradation in an Applied Biosystems model 477A/120A protein sequencing system.

Analysis of Conformational Changes by Limited Proteolysis—Increasing amounts of heparin, hyaluronic acid, chondroitin sulfate, or collagen were added to PEDF (2 μg), and the sam-
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including tetrasaccharides (dp4), octasaccharides (dp8), and hexadecasaccharides (dp16) in 100 µL of TBS for 1 h before the addition to the wells. Wells were developed as described above and all data points were collected in duplicates.

RESULTS

The Susceptibility of PEDF to Limited Trypsin Proteolysis Was Increased by Heparin—To investigate putative structural changes induced by ligand binding, PEDF was titrated with heparin, hyaluronic acid, chondroitin sulfate, or collagen and studied by means of limited proteolysis. In the presence of hyaluronic acid, chondroitin sulfate, and collagen, PEDF remained resistant to proteolysis (data not shown). However, heparin significantly modified the proteolytic susceptibility, and PEDF was readily cleaved into smaller fragments (Fig. 1). One fragment of 29 kDa migrated as a fuzzy band, and two smaller fragments of ~23 kDa migrated as a closely spaced doublet. At increasing amounts of heparin, the rate of proteolysis was accelerated, as observed by a decrease in staining intensity of the band representing intact PEDF. The specificity of the interaction was verified by including heparinase in the reaction mixture prior to trypsin digestion, which reversed the effect of heparin (Fig. 1). Collectively, these data are consistent with a change in the PEDF structure following binding of heparin.

Identification of the Initial Trypsin Cleavage Site—The PEDF fragments produced by limited digestion with trypsin were characterized by automated Edman degradation. The 29-kDa fragment produced the sequence Glu-Ile-Pro-Asp-Glu, suggesting that this band represents the C-terminal part of PEDF spanning Glu179–Pro399 (theoretical molecular mass 25 kDa) (Figs. 1 and 2). The fragment includes the N-linked glycan at Asn266 (7), and the fuzzy appearance in SDS-PAGE is likely caused by heterogeneity of the attached glycan (~2 kDa) and/or C-terminal truncation at Arg380, Lys391, or Arg397. The lower 23-kDa closely spaced doublet did not sequence during Edman degradation, suggesting that these fragments represent the pGlu-blocked N terminus of PEDF (7). The theoretical molecular mass of the pGlu1–Lys178 fragment (20 kDa) corresponds well with the observed mass of 23 kDa (Fig. 1). The appearance of a doublet is most likely caused by C-terminal truncation.

FIGURE 2. Structural representation of PEDF. Schematic representation of PEDF (not to scale) depicts the location of the initial trypsin cleavage (Lys178–Glu179), as confirmed by Edman degradation. The residues involved in heparin binding (Lys127, Lys128, and Arg130) are indicated, as well as the three tryptophan residues present in the protein, including the surface-exposed Trp164.

FIGURE 3. Proteolytic fragmentation of PEDF is dependent on ionic strength. PEDF (2 µg) was subjected to proteolysis by trypsin (1:40 w/w ratio) in the presence of increasing NaCl (mM) concentrations as indicated. The reactions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The position of intact PEDF is shown by an arrow on the left. A reduction in ionic strength causes PEDF to become susceptible to proteolysis.

FIGURE 4. An increase of ionic strength reverts the heparin-induced conformational change of PEDF. PEDF (2 µg) was incubated with trypsin (2 µg) and heparin (0.2 µg) in the presence of increasing concentration of NaCl (mM) as indicated. Trypsin (2 µg) and PEDF (2 µg) are included as controls. Reaction products were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The result suggests that the addition of salt disrupts heparin binding and renders PEDF less prone to proteolysis.
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The Proteolytic Susceptibility of PEDF Is Affected by Ionic Strength—Because the interaction between PEDF and heparin is likely to be electrostatic in nature (12), we tested whether the proteolytic susceptibility of PEDF was affected by the ionic strength of the buffer. In the presence of 100 mM NaCl the protein was resistant to cleavage by trypsin. However, in the absence of NaCl, PEDF was completely digested (Fig. 3). The digestion of PEDF at low ionic strength generated fragments similar to those observed during the digestion at physiological conditions in the presence of heparin (Fig. 1). The increase in proteolytic susceptibility at low ionic strength could be reversed by the addition of NaCl (data not shown). This observation shows that the overall structure of PEDF is maintained under all conditions. In the presence of 100 mM NaCl the [urea]_{50%} value is 3.30 ± 0.02 M and the m_{D-N} value is 2.11 ± 0.14 M⁻¹, corresponding to a calculated stability (ΔG_{D-N}) of 6.97 ± 0.63 kcal/mol (Fig. 5B). This value is within the range of typical globular proteins (5–15 kcal/mol) (21). When heparin was included in the analysis, the [urea]_{50%} value was slightly reduced to 3.03 ± 0.04 M with an m_{D-N} value of 1.97 ± 0.21 M⁻¹, corresponding to a stability of 5.97 ± 0.64 kcal/mol (Fig. 5B). In the absence of NaCl, the [urea]_{50%} value was further reduced to 2.47 ± 0.03 M (m_{D-N} value of 2.00 ± 0.17 M⁻¹), corresponding to 4.94 ± 0.42 kcal/mol (Fig. 5B). These findings suggest that the increase in proteolytic susceptibility observed at low ionic strength or in the presence of heparin at physiological conditions is the result of a change, rendering PEDF receptive to limited proteolysis. To investigate whether the two circumstances generated similar structural alterations, PEDF was titrated with NaCl in the presence of heparin and analyzed by reduced SDS-PAGE following tryptic digestion (Fig. 4). It was apparent that an increase in the ionic strength reversed the effect of heparin and caused PEDF to become resistant to proteolysis at higher NaCl concentrations. Because the structural change induced by heparin is reverted by an increase in the salt concentration, it is likely that the structural change induced by heparin and low ionic strength is similar in nature. This is further supported by the fact that appearance of identical reaction products are obtained following limited proteolysis of PEDF at low ionic strength and in the presence of heparin (Figs. 1 and 3).

Structural Analysis of PEDF Using Intrinsic Tryptophan Fluorescence and CD Spectroscopy—To investigate the influence of heparin and ionic strength on the tertiary structure, we subjected PEDF to urea denaturation and monitored the structural impact by Trp fluorescence spectroscopy. The degree of unfolding was evaluated by the ratio of fluorescence at 335 nm (hydrophilic environment) and 355 nm (hydrophobic environment), determined to be the wavelengths of maximum intensity for native and fully unfolded PEDF, respectively (Fig. 5A). The fluorescence spectra of PEDF in the presence of (i) 100 mM NaCl, (ii) heparin and 100 mM NaCl, and (iii) 0 mM NaCl in the absence of denaturant were similar (data not shown). This observation shows that the overall structure of PEDF is maintained under all conditions. In the presence of NaCl, PEDF was titrated with NaCl and urea (Fig. 5B). The far-UV CD spectra of PEDF (4 μM) in the presence and absence of NaCl (100 mM) and heparin (2 mg/ml) were similar (not shown). This observation shows that the over-all structure of PEDF is maintained under all conditions. The [urea]_{50%} value was 3.30 ± 0.02 M and the m_{D-N} value was 2.11 ± 0.14 M⁻¹, corresponding to a calculated stability (ΔG_{D-N}) of 6.97 ± 0.63 kcal/mol (Fig. 5B). This value is within the range of typical globular proteins (5–15 kcal/mol) (21). When heparin was included in the analysis, the [urea]_{50%} value was slightly reduced to 3.03 ± 0.04 M with an m_{D-N} value of 1.97 ± 0.21 M⁻¹, corresponding to a stability of 5.97 ± 0.64 kcal/mol (Fig. 5B). In the absence of NaCl, the [urea]_{50%} value was further reduced to 2.47 ± 0.03 M (m_{D-N} value of 2.00 ± 0.17 M⁻¹), corresponding to 4.94 ± 0.42 kcal/mol (Fig. 5B). These findings suggest that the increase in proteolytic susceptibility observed at low ionic strength or in the presence of heparin at physiological conditions is the result of a
localized structural change and not a global unfolding of the PEDF structure. This conclusion was further supported by CD spectroscopy, which revealed no significant change in the secondary structure of PEDF in the absence of NaCl or presence of heparin (Fig. 5C).

Heparin-derived Oligosaccharides Smaller than 8 Disaccharide Units (dp16) Do Not Bind to PEDF nor Induce a Conformational Change—The ability to induce a conformational change is likely to depend on the size of the heparin. To investigate this, PEDF was subjected to limited proteolysis in the presence of intact heparin or heparin-derived oligosaccharides, including tetrasaccharides (dp4), octasaccharides (dp8), and hexadecasaccharides (dp16). Only intact heparin and the hexadecasaccharides (dp16) accelerated proteolysis, consistent with a conformational change (Fig. 6). The tetrasaccharides (dp4) and octasaccharides (dp8) were apparently unable to coordinate the intramolecular interactions required for the conformational change to occur.

To investigate whether the tetra- and octasaccharides were able to bind PEDF without inducing a conformational change as observed for intact heparin and the hexadecasaccharides, we used a heparin-based ELISA. As expected, PEDF bound intact heparin in a dose-dependent manner (Fig. 7A). The binding of the oligosaccharides was evaluated in a competition format. Thus, the binding capacity of PEDF was reduced in the presence of intact heparin or hexadecasaccharides (dp16) (Fig. 7B). Conversely, the tetrasaccharides (dp4) and octasaccharides (dp8) did not prevent the binding of the PEDF to the heparin-coated microtiter wells (Fig. 7B). Collectively, these data suggest that only intact heparin and the hexadecasaccharide (dp16) were able to bind to PEDF and induce a conformational change.

DISCUSSION

The binding of PEDF to a receptor on the cell surface of retinoblasoma Y-79 cells is significantly improved by the presence of heparin (14). It has been suggested that the mechanism of enhanced receptor binding involves a structural change of PEDF (14). In this study, we have employed biochemical and biophysical methods to investigate this hypothesis. Limited proteolysis has previously been used as a tool to investigate the inhibitory capacity of bovine PEDF (3, 22). These studies concluded that bovine PEDF was resistant to proteolysis and only a single cleavage of Arg360–Leu361 within the reactive center loop was detected when the protein was treated with trypsin in the presence of 150 mM NaCl (3). As shown in this study, human PEDF is not cleaved, using similar conditions, most likely because the homologous peptide bond, His362–Leu363, is not a substrate for trypsin. However, we observed that PEDF became a substrate for trypsin in the presence of heparin, suggesting that the protein undergoes a conformational change. The addition of heparinase reversed this effect, supporting the fact that the altered proteolytic susceptibility was caused by the heparin-PEDF interaction. Interestingly, the interaction between PEDF and other identified binding partners, including type I collagen (7, 9, 10) and glycosaminoglycans such as hyaluronic acid and chondroitin sulfate (12), did not increase the proteolytic susceptibility. The fragments generated by limited trypsin digestion were analyzed by automated Edman degradation. This revealed that the initial cleavage event occurred at Lys178–Glu179, a tryptic cleavage site which has also been reported using recombinant PEDF (23). This result is consistent with an increased segmental mobility or a local unfolding in that region upon heparin binding.

In addition to the presence of heparin, we also observed that low ionic strength increased the proteolytic susceptibility. However, the stability was recovered by the addition of NaCl, suggesting that the impact on the structure was reversible and not the result of a structural collapse. To test whether a similar effect of ionic strength was a property of other noninhibitory serpins in general, we subjected ovalbumin to trypsin digestion in the absence or presence of 100 mM NaCl. Unlike PEDF, ovalbumin was stable at both conditions (data not shown). Although the absence of NaCl represents a nonphysiological condition, we hypothesize that the observed increase in proteolytic sensitivity at low salt concentrations or in the presence of heparin reflects similar PEDF conformations. This was substantiated by (i) the appearance of similar reaction products after limited trypsin digestion and (ii) the observation that the heparin-induced conformational change was reversed when ionic strength was increased.

4 Numbering of bovine PEDF is done by homology to the mature N terminus of the human protein: Gln1-Asn-Ala-.....-Gly-Thr397.
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Analysis of the structure by intrinsic tryptophan fluorescence at nondenaturing conditions and by CD spectroscopy showed that the gross structure of PEDF was maintained in buffers containing 0 mM NaCl, 100 mM NaCl, or 100 mM NaCl and heparin. However, the urea equilibrium denaturation studies suggest that PEDF was destabilized in the presence of heparin or in the absence of NaCl. It is thus apparent that the changes induced by heparin or low ionic strength do not alter the overall structure of the protein. The structural changes evident by limited proteolysis are therefore likely to be subtle and possibly localized to the spatial region around Lys<sup>178</sup>. The absence of any change in secondary structure, as observed by CD spectroscopy, is in agreement with the fact that the Lys<sup>178</sup>–Glu<sup>179</sup> bond is localized in an unstructured loop region, connecting helix F and strand 3 of β-sheet A (2). Interestingly, Trp<sup>163</sup> is the only surface-exposed tryptophan residue found in PEDF and localized in helix F in the spatial vicinity of both the heparin binding site (Lys<sup>127</sup>, Lys<sup>128</sup>, Arg<sup>130</sup>) and Lys<sup>178</sup> (2, 11). The absence of any detectable change in intrinsic tryptophan fluorescence by the addition of heparin or the absence NaCl supports the notion that the change observed by limited digestion is subtle and local. However, it is likely that this structural perturbation supports the interaction between PEDF and a cell surface receptor, as suggested by Alberdi et al. (14). The finding that low ionic strength induces a similar structural change is not clear. However, we can assume that PEDF is locked in a stable conformation by interactions present at physiological conditions. These interactions are disrupted by low ionic strength, and the result is a relaxed and destabilized conformation of PEDF that is susceptible to trypsin cleavage. And is similar or identical to those disrupted by the binding of heparin, these interactions are without physiological relevance.

It is evident that particular structural requirements of the heparin molecule exist because derivatives smaller than the hexadecasaccharide (dp16) failed to bind and induce the structural perturbation of the PEDF structure. This finding links binding ability with the ability to change structurally.

The functional activity of PEDF is very potent and is likely tightly controlled. It has been proposed previously that this activity can be regulated by the binding of PEDF to interaction partners in the extracellular matrix (24). This was further substantiated by the finding that the binding of PEDF to a cell surface receptor could be enhanced by the presence of heparin sulfate (14). Here we show that PEDF is indeed a flexible protein that adopts a different structure upon binding of heparin. Our data indicate that the variability of PEDF functions can be regulated by this structural alteration. This mechanism may be involved in controlling the activity of a relatively high concentration of PEDF found in plasma (100 nm) (7) by allowing physiological activity to be expressed only in environments containing heparin/heparan sulfate.

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