Articles

Dual-Site Recognition of Different Extracellular Matrix Components by Anti-Angiogenic/Neurotrophic Serpin, PEDF

Norihisa Yasui,[†] Terumi Mori,[†] Daisuke Morito,[‡] Osamu Matsushita,[§] Hiroki Kourai,[†] Kazuhiro Nagata,[‡] and Takaki Koide*,[†]

Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima 770-8506, Japan, Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8397, Japan, and Department of Microbiology, Faculty of Medicine, Kagawa Medical University, Miki-cho, Kitagun, Kagawa 761-0793, Japan

Received November 13, 2002

ABSTRACT: Pigment epithelium-derived factor (PEDF), a member of the serine protease inhibitor (serpin) superfamily, possesses anti-angiogenic and neurotrophic activities. PEDF has been reported to bind to extracellular matrix (ECM) components such as collagens and glycosaminoglycans (GAGs). In this study, to determine the binding sites for collagens and GAGs, we analyzed the interaction of recombinant mouse PEDF (rPEDF) with collagen I and heparin. By utilizing residue-specific chemical modification and site-directed mutagenesis techniques, we revealed that the acidic amino acid residues on PEDF (Asp^{255}, Asp^{257}, and Asp^{299}) are critical to collagen binding, and three clustered basic amino acid residues (Arg^{145}, Lys^{146}, and Arg^{148}) are necessary for heparin binding. Mapping of these residues on the crystal structure of human PEDF (Simonovic, M., Gettins, P. G. W., and Volz, K. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 11131−11135) demonstrated that the collagen-binding site is oriented toward the opposite side of the highly basic surface where the heparin-binding site is localized. These results indicate that PEDF possesses dual binding sites for different ECM components, and this unique localization of ECM-binding sites implies that the binding to ECM components could regulate PEDF activities.

Extracellular matrix (ECM)[1] is a complex network of many proteins, proteoglycans, and glycosaminoglycans (GAGs) that not only provides mechanical strength for cells and tissues but also regulates the cell proliferation and differentiation by regulating growth factor activities (*1*, *2*).

Pigment epithelium-derived factor (PEDF) is a 50-kDa glycoprotein that belongs to the serine protease inhibitor (serpin) superfamily (*3*) but does not inhibit any proteases tested to date (*4*). PEDF was initially identified in cultured human fetal pigment epithelium as a neurotrophic factor possessing neuronal differentiating activity against Y-79 retinoblastoma cells (*5*). Recent reports have demonstrated its neurotrophic activity using other cell systems: PEDF also exhibits neurotrophic activities in primary cultures of cerebellar granule cells (*6*, *7*) and promotes the survival and differentiation of developing spinal motor neurons (*8*, *9*).

PEDF also displays an anti-angiogenic activity in the eye (*10*). It acts on endothelial cells that are forming new vessels and induces their apoptosis without harming the preexisting vasculature (*11*). Recently, Volpert et al. demonstrated that PEDF derives this specificity for activated endothelial cells from their dependence on Fas/Fas ligand-mediated apoptosis (*12*).

Although it has been reported that PEDF binds to retinoblastoma cells, cerebellar granule cells (*13*), and the neural retina (*14*), a PEDF receptor has not been identified. Moreover, the mechanisms explaining these PEDF activities are not well-understood.

PEDF interacts with components of the ECM, such as GAGs (*15*, *16*) and collagens in vitro (*17*). It is therefore speculated that the interaction of PEDF with different ECM components could allow for different PEDF activity (*18*). Interestingly, a recent report suggests that maspin, another anti-angiogenic serpin (*19*), also binds to collagen types I and III (*20*). The similarity of this collagen-binding property suggests that collagen binding may be a common character of anti-angiogenic serpins. Heat shock protein 47 (HSP47), a procollagen-specific molecular chaperone, is also known as a collagen-binding serpin, and its collagen-binding profile has recently been characterized (*21*).

Recently, the crystal structure of recombinant human PEDF has been solved (*22*). PEDF has a unique asymmetric

* To whom correspondence should be addressed. Tel: +81-88-656-7521. Fax: +81-88-656-7521. E-mail: tkoide@bio.tokushima-u.ac.jp.
[†] The University of Tokushima.
[‡] Kyoto University.
[§] Kagawa Medical University.

[1] Abbreviations: ECM, extracellular matrix; CBB, Coomassie Brilliant Blue; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GAGs, glycosaminoglycans; GSH, glutathione; GST, glutathione-*S*-transferase; HBST, HEPES-buffered saline with 0.05% Tween20; HSP47, heat shock protein 47; PEDF, pigment epithelium-derived factor; serpin, serine protease inhibitor; standard three-letter and one-letter abbreviations were used for common amino acids.

10.1021/bi0206558 CCC: $25.00
Published on Web 03/01/2003

charge distribution, that is, the basic residues are concentrated on one surface, and the acidic residues are concentrated on the opposite surface (*22*). It has been proposed that GAGs/heparin-binding site is located on the basic surface of the molecule (*15*, *22*).

In this paper, we show that PEDF has two distinct binding sites for different ECM components such as collagens and heparin. We have mainly used residue-specific chemical modification and site-directed mutagenesis techniques to determine the ECM-binding sites on PEDF. The determined localization of collagen- and heparin-binding sites suggests that PEDF interacts differently with each ECM component and implies that the biological activities of PEDF could be differentially regulated by the interaction with collagens and GAGs.

EXPERIMENTAL PROCEDURES

DNA Manipulations and Sequencing. Mouse PEDF (accession no. AF017057) was cloned from a mouse E 15.5 day SuperScript cDNA Library (GIBCO BRL) using polymerase chain reaction (PCR) with the following oligonucleotides: CCGGAATTCCACGGCAGCAGCCAGAACGTCCCCA and ACGCGTCGACTTAAGTGCTACTGGGGTCCAGGATTC. PCR products were subcloned into the vector pT7 Blue T (Novagen) and then digested with *Eco*RI and *Sal*I. The resulting fragment was inserted into pGEX-5X-1 (Amersham Bioscience, Inc.) to construct a full-length PEDF/pGEX-5X-1 expression vector. To obtain the nucleotide fragment encoding the mature form of PEDF (Asn^{21}−Thr^{417}; amino acid numbering is from the precursor polypeptide sequence), PCR was performed using either full-length PEDF/pGEX-5X-1 as a template and the following primers: GGAATTCAACGTCCCCAGCAGCTCTGA and ACGCGTCGACTTAAGTGCTACTGGGGTCCAGG. PCR products were digested with *Eco*RI and *Sal*I and subcloned into pGEX-5X-1 to construct the PEDF/pGEX-5X-1 expression vector.

To introduce point mutations, PCR was carried out by the procedure of Higuchi et al. (*23*) using PEDF/pGEX-5X-1 as a template and an appropriate pair of overlapping mutagenic primers (see Supporting Information) and pGEX primers. The resulting mutated fragments were digested with appropriate sets of restriction enzymes and were used to replace the corresponding fragments in PEDF/pGEX-5X-1. All constructs were sequenced to confirm the correct reading frame and the mutation on an automated nucleotide sequencer (model ABI PRISM 377, Perkin-Elmer Life Science, Foster City, CA).

Purification of Recombinant Proteins. Escherichia coli JM109-competent cells were transformed with the recombinant plasmids constructed as above. Each clone was grown in 125−500 mL of LB medium to an optical density at 600 nm of 1−2. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM, and cells were grown for a further 12−16 h at 25 °C.

Purification of the GST fusion proteins was performed on a glutathione (GSH)-Sepharose 4B affinity column (Amersham Biosciences, Inc.) as described by the manufacturer. To obtain the GST-cleaved forms of the recombinant proteins, Factor Xa (Amersham Biosciences, Inc.) cleavage on beads was performed in the Factor Xa cleavage buffer (50 mM HEPES·Na (pH 7.5), 100 mM NaCl, and 1 mM $CaCl_2$) following the manufacturer's protocol. The purified proteins were stored at −80 °C until needed.

Solid-Phase Binding Assay. Immobilization of Collagen I (Cellmatrix TypeI-C, Nitta Gelatin, Osaka, Japan) onto CNBr-activated Sepharose 4B (Amersham Biosciences, Inc.) or NHS-activated Sepharose 4FF (Amersham Biosciences, Inc.) was performed as described (*24*). Heparin Sepharose CL-6B (Amersham Biosciences, Inc.) was diluted with mock-coupled Sepharose 4B to make the binding capacity for rPEDF nearly equal to that of collagen beads. The binding of rPEDF to collagen I and heparin was examined by the solid-phase binding assay as described elsewhere (*24*). Briefly, *E. coli* lysate containing the GST−PEDF fusion protein, or 3−5 μg of purified protein was mixed with the binding buffer (20 mM HEPES·Na (pH 7.5), 100 mM NaCl, and 0.05% Tween20) and a 30-μL bed of the affinity beads. When necessary, the NaCl concentration of the binding buffer was modified to 150 mM. The binding reaction was carried out at 4 °C for 1 h. After washing the bed, proteins retained on the beads were eluted by adding Laemmli's SDS sample buffer and separated by 10 or 12% SDS−polyacrylamide gel electrophoresis (SDS−PAGE). Proteins were visualized by Coomassie Brilliant Blue (CBB) staining.

Washing Elution Study of GST−PEDF Bound to Collagen I Affinity Beads. GST−PEDF or GST−HSP47 (*25*) fusion protein bound to collagen beads (20-μL bed) was washed twice with 400 μL of binding buffer ranging in NaCl concentration from 50 to 400 mM. After washing, the proteins retained on the beads were eluted with Laemmili's SDS sample buffer and analyzed by SDS−PAGE.

Binding Competition Assay. The PEDF-binding assay in the presence of heparin was performed as follows. Purified GST−PEDF fusion protein (5 μg) and affinity beads (30-μL bed) were mixed with 200 μL of binding buffer containing heparin (Sigma), and the mixture was rotated at 4 °C for 1 hr. The beads were washed twice with binding buffer. The protein retained on the beads was eluted with Laemili's SDS sample buffer and analyzed by SDS−PAGE.

Chemical Modification of Collagen I. Chemical modification of collagen I was performed as described previously (*21*), except for Asp and Glu modifications. For Asp and Glu, the collagen I immobilized to NHS-activated Sepharose 4FF was treated with 150 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 1 M Tris-HCl (pH 7.4) at room temperature for 6 h.

Chemical Modification of rPEDF. The preparation of chemically modified rPEDF was as follows. For Asp and Glu modification, purified rPEDF was treated with 0.1 M EDC and 0.1 M ethanolamine in Factor Xa cleavage buffer (113 μL) at room temperature for 30 min. Then, 3.3 μL of 3 M EDC was supplied, and the mixture was further incubated at room temperature for 30 min. The reaction was stopped by passing the reaction mixture through a BioGel P-10 fine gel filtration column (BioRad) equilibrated in HEPES-buffered saline with 0.05% Tween20 (HBST). For Arg modification, rPEDF was incubated with 5 mM 2,3-butanedione in 35 mM borate buffer (pH 8.3) at room temperature for 90 min. The reaction was stopped by passing the reaction mixture through the BioGel P-10 fine gel filtration column equilibrated in 0.1 M borate buffer (pH 8.3). To acetylate amino groups of Lys residues, purified rPEDF was treated with 60 mM acetic anhydride in Factor

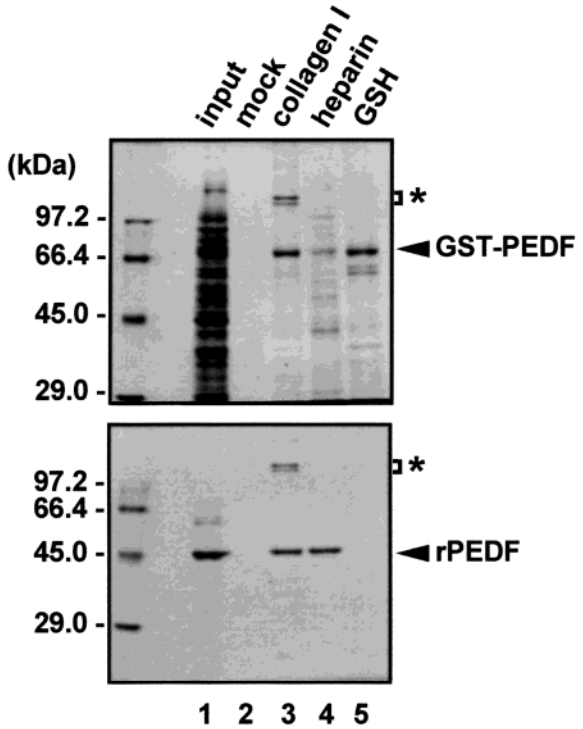

FIGURE 1: Interaction of recombinant PEDF proteins with collagen I and heparin. Interaction of GST−PEDF fusion protein (upper panel) and GST-cleaved form of PEDF (lower panel) with immobilized collagen I and heparin. After washing the affinity beads, proteins retained on the beads were eluted and separated on SDS−polyacrylamide gel and visualized by CBB staining. Asterisk (*) indicates a chain of collagen I eluted from collagen beads.

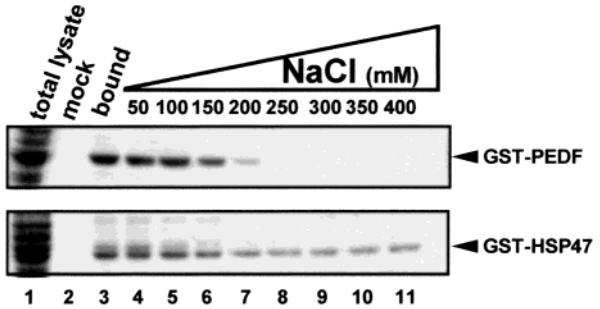

FIGURE 2: Contribution of ionic interaction to PEDF−collagen binding. GST−PEDF or GST−HSP47 bound to collagen−sepharose was washed with a buffer ranging in NaCl concentration from 50 to 400 mM. Proteins retained on beads were eluted and separated on a 10% SDS−polyacrylamide gel and visualized by CBB staining.

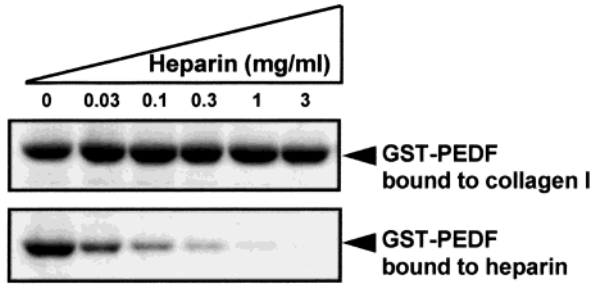

FIGURE 3: PEDF possesses a binding site for collagen different from that for heparin. GST−PEDF fusion protein and affinity beads were mixed with buffers containing varying concentrations of heparin, and the GST−PEDF retained on beads was separated on a 10% SDS−polyacrylamide gel and visualized by CBB staining. GST−PEDF bound on collagen beads (upper panel) and on heparin beads (lower panel) is shown.

Xa cleavage buffer at room temperature for 15 min. The reaction mixture was then treated with 100 mM Tris-HCl (pH 7.4) to quench excess acetic anhydride and diluted with HBST for the solid-phase binding assay as described above.

Circular Dichroism (CD). CD spectra of rPEDF and the PEDF mutants were recorded on a Jasco J-820 spectropolarimeter at 25 °C using a cell of 0.1-cm path length. The concentrations of proteins used were 0.127−0.341 mg/mL in 25 mM HEPES-Na (pH 7.5), 75 mM NaCl, and 0.5 mM $CaCl_2$.

RESULTS

PEDF Binds to Collagen I Mainly by Ionic Interaction. Previous studies have shown that PEDF binds to GAGs/heparin (*15*, *16*) and collagens I and III but not to collagens II and IV (*17*). The association of recombinant GST−PEDF fusion protein with collagen I and heparin was first confirmed by solid-phase binding assay using *E. coli* lysate containing GST−PEDF. As shown in Figure 1, GST−PEDF was specifically purified from the crude lysate using collagen I beads (Figure 1, upper panel, lane 3). This specific interaction of PEDF with collagen I was also demonstrated using the purified and GST-cleaved form of rPEDF (Figure 1, lower panel, lane 3). Therefore, GST−PEDF and rPEDF are confirmed to be available in the analyses of the PEDF−collagen interaction. On the other hand, we could hardly extract the specific binding to heparin when using crude bacterial lysate containing GST−PEDF (Figure 1, upper panel, lane 4) because of nonspecific binding of proteins derived from *E. coli* to heparin. For this reason, heparin binding of PEDF was examined using purified protein in this study (Figure 1, lower panel, lane 4).

To evaluate the contribution of ionic interaction to the binding of PEDF to collagen I, GST−PEDF bound to collagen−Sepharose was washed with buffer ranging in NaCl concentration from 50 to 400 mM. Figure 2 shows SDS−PAGE analysis of GST−PEDF retained on the collagen beads. The amount of GST−PEDF on collagen−Sepharose decreased as the NaCl concentration in the wash buffer increased (Figure 2, upper panel). It should be noted that a significant amount of GST−PEDF was retained on collagen beads even under the physiological conditions of 150 mM NaCl (Figure 2, lane 6). As a control experiment, we carried out a similar assay using GST−HSP47 fusion protein (*25*). HSP47, another member of the serpins, is a procollagen-specific molecular chaperone and is known to bind to at least types I−V collagen (*26*). The binding of GST−HSP47 to collagen I was not largely affected by the NaCl concentration in the buffer (Figure 2, lower panel), consistent with a previous observation (*27*). These results indicate that ionic interaction largely contributes to the binding of PEDF to collagen I as well as to heparin (*15*).

Collagen-Binding Site on PEDF Is Distinct from its Heparin-Binding Site. To determine whether PEDF binds to collagen I with the same binding site for heparin or not, the binding of GST−PEDF to immobilized collagen I or heparin was examined in the presence of heparin as a competitor. As shown in Figure 3, the binding of GST−PEDF to heparin−Sepharose was competed by heparin in a

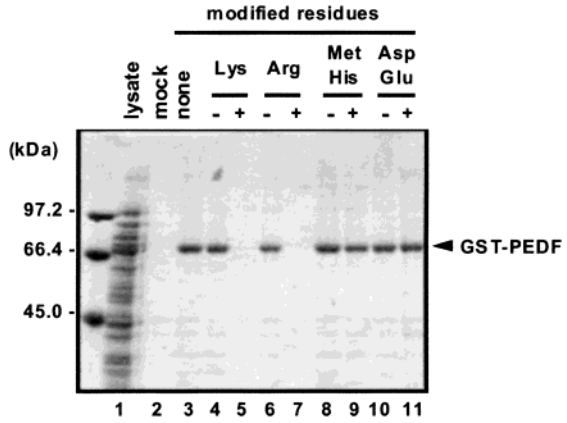

FIGURE 4: Effect of chemical modification of collagen on PEDF−collagen interaction. Binding of GST−PEDF to the immobilized collagen I with modified residues was analyzed by solid-phase binding assay. Proteins retained on beads were separated on a 10% polyacrylamide gel and visualized by CBB staining. Lanes for mock-modified (that is, treated only with buffers) collagens are indicated by −. An arrowhead indicates GST−PEDF fusion protein.

concentration-dependent fashion (Figure 3, lower panel), while the binding of GST−PEDF to collagen−Sepharose was not competed by heparin (Figure 3, upper panel). These observations suggest that PEDF binds to collagen I using a different binding site from that for heparin.

Both Lys and Arg Residues on Collagen Are Important for PEDF Binding. To determine the amino acid residues on collagen to be recognized by PEDF, a similar solid-phase binding assay was carried out using chemically modified collagen I. When Lys or Arg residues were modified with acetic anhydride or 2,3-butanedione, respectively, the binding of GST−PEDF to collagen I was abolished (Figure 4, lanes 4−7). Other amino acid residues on collagen I such as His, Met, Asp, and Glu did not contribute markedly to the PEDF−collagen interaction (Figure 4, lanes 8−11). Similar results were obtained using chemically modified collagen III (data not shown). These results suggest that both Lys and Arg residues are accommodated in the PEDF-binding site on collagen.

Effect of Chemical Modification of rPEDF on its ECM Binding. Since an ionic interaction largely contributes to the binding of PEDF to collagen I (Figure 2), and both Lys and Arg residues on collagen are important in PEDF−collagen interaction (Figure 4), it was expected that negatively charged residues on PEDF are important in the collagen binding. To confirm this, the carboxyl groups of Asp and Glu residues on rPEDF were chemically blocked with EDC and ethanolamine, and the binding of modified rPEDF to collagen was examined. As shown in Figure 5A, on the modification of Asp and Glu residues, PEDF lost its collagen-binding activity. However, this modification did not affect the heparin binding. These results indicate that acidic amino acid, Asp and/or Glu residues, are important in the PEDF−collagen interaction.

Generally, the ionic interaction of proteins with GAGs/heparin is mediated by positively charged residues of proteins and negatively charged groups of GAGs/heparin. It was reported earlier that partial biotinylation of Lys residues on PEDF prevents its heparin binding (*15*). For a finer assessment of the contribution of basic residues on PEDF to the heparin binding, Lys and Arg residues were independently

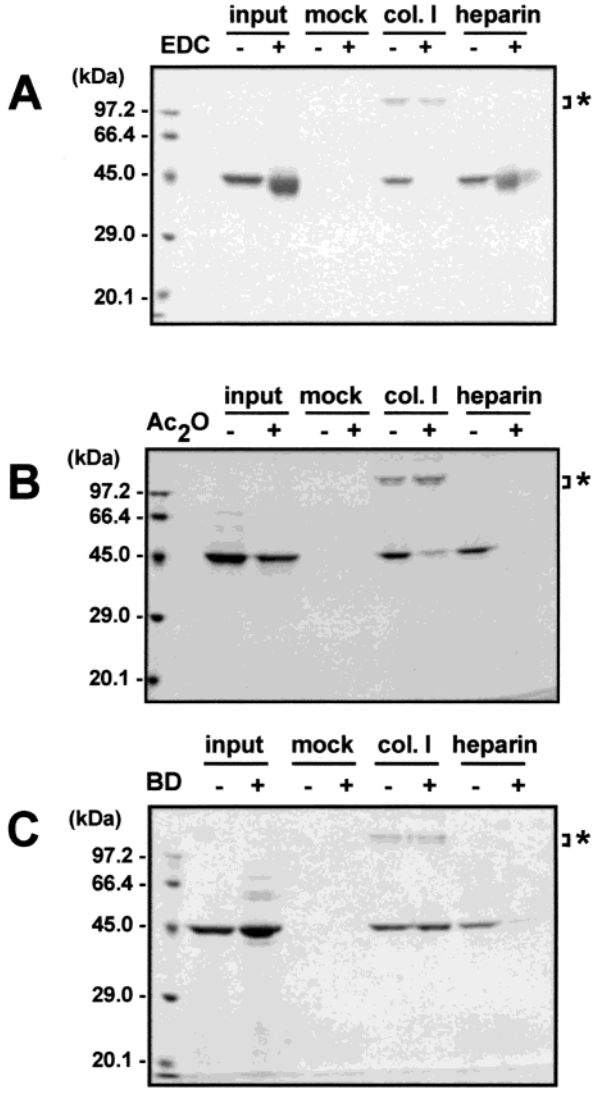

FIGURE 5: Effect of chemical modification of rPEDF on the binding to collagen I and heparin. rPEDF was treated with various residue-specific modification reagents, and the binding to collagen and heparin was examined. Lanes for rPEDF treated without chemical reagents are indicated by −. (A) Asp and Glu on rPEDF were modified with EDC and ethanolamine. (B) Lys on rPEDF were modified with acetic anhydride (Ac_2O). (C) Arg residues on rPEDF were modified with 2,3-butandione (BD). Asterisk (*) indicates a chain of collagen I eluted from collagen beads.

modified with residue-specific reagents. The ECM-binding activity of the modified rPEDF was also examined by a solid-phase binding assay. As shown in Figure 5B, rPEDF with acetylated Lys residues lost heparin-binding affinity, while the collagen-binding activity was retained. The Arg modification of rPEDF also abolished the heparin-binding activity but little affected the collagen binding (Figure 5C). These results suggest that both Lys and Arg residues of PEDF are essential for heparin binding.

Determination of the Collagen- and Heparin-Binding Sites on PEDF by Site-Directed Mutagenesis. The chemical modification studies indicated that Asp and/or Glu residues on PEDF are essential for the collagen binding, and both Lys and Arg residues significantly contribute to the heparin binding (Figure 5). It should be noted that the PEDF molecule has an asymmetric charge distribution on its surface (*15*, *22*). Therefore, we next mutated all of the Asp and Glu residues on the positively charged surface (ref *22*, Figure 8,

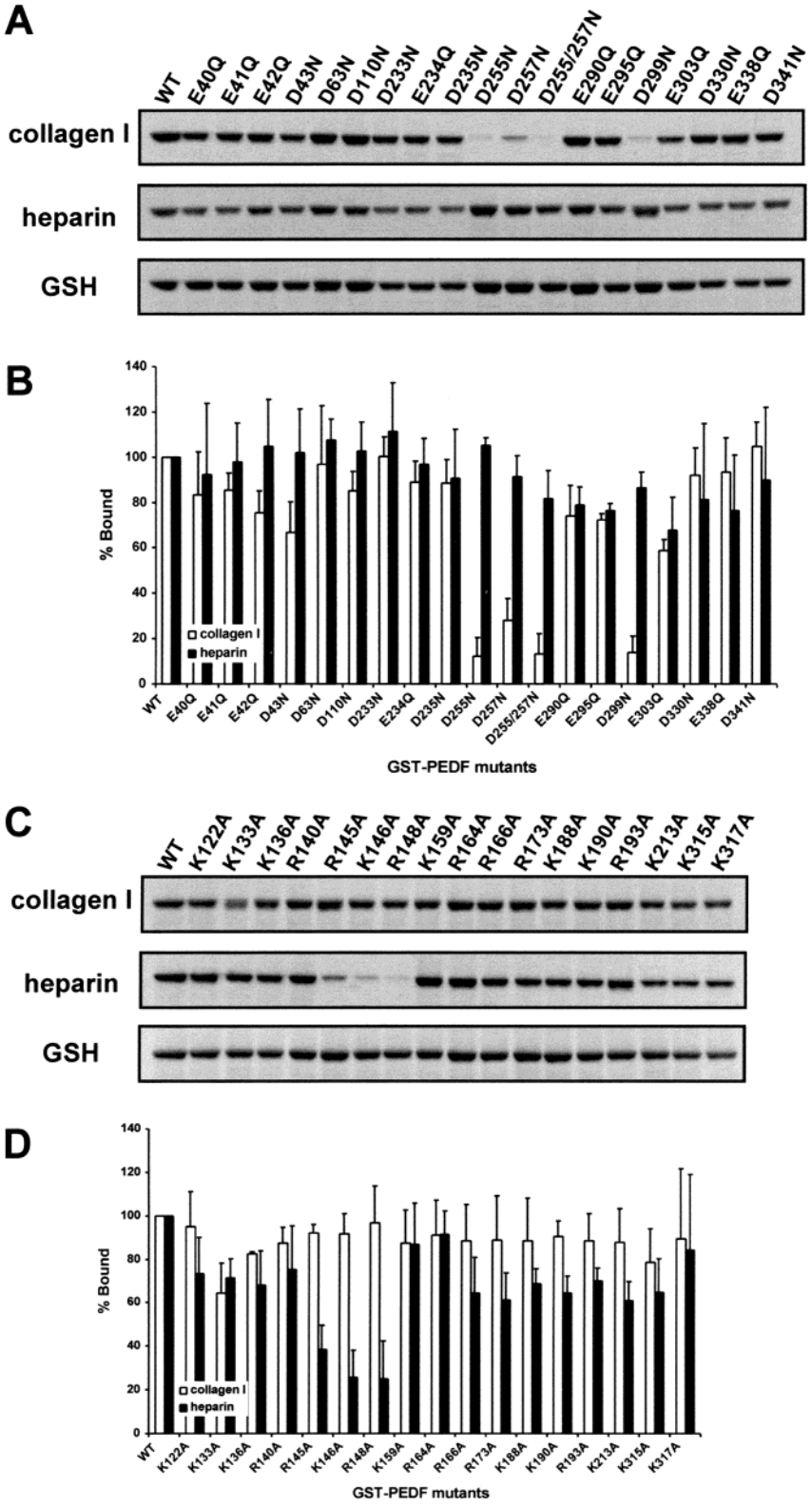

FIGURE 6: Binding profile of GST−PEDF mutants to collagen I and heparin. The interaction of GST−PEDF point mutants with ECM components was analyzed by the solid-phase binding assay. Four micrograms of GST−PEDF mutant protein and a 30-μL bed of beads were used. (A) Binding of GST−PEDF mutants altered Asp and Glu to collagen I (top panel), heparin (middle panel), and GSH (bottom panel). (B) Amount of GST−PEDF mutants in part A was quantified by densitometry using Scion Image. The intensity in each band of the GST−PEDF mutants bound to collagen I or heparin was corrected for the amount of each protein bound to GSH and normalized to the wild-type GST−PEDF. Values are mean ± SD ($n = 3$). (C) Binding of GST−PEDF mutants altered Lys and Arg residues to collagen I (top panel), heparin (middle panel), and GSH (bottom panel). (D) Amount of GST−PEDF mutants in part C was quantified by densitometry. Corrections of the intensity in each band were made similarly to part B. Values are mean ± SD ($n = 3$).

left), and all of the Lys and Arg on the opposite surface of PEDF (Figure 8, right) to map each ECM-binding site. Most of the mutant proteins could be readily obtained as soluble forms, and these mutants were subjected to the ECM-binding assay. Only one PEDF mutant protein (E289Q) could not be obtained because of a failure in folding. When compared

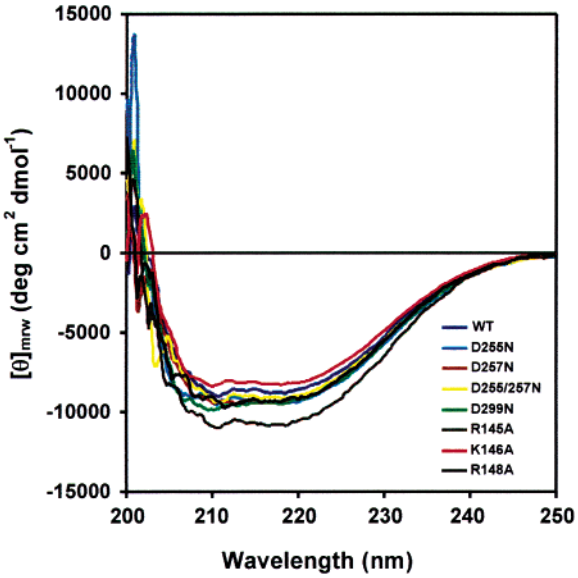

FIGURE 7: CD spectra of rPEDF and its mutant forms reduced in ECM-binding activity. The CD signal is expressed as mean molar residue ellipicity.

with wild-type rPEDF, the D255N and D299N mutants showed a marked reduction in collagen-binding activity (Figure 6A, top panel). A moderate but significant reduction in collagen-binding activity was also observed for the D257N mutant (Figure 6A, top panel). No loss of heparin-binding activity was observed for these acidic amino acid mutants (Figure 6A, middle panel). We thus conclude that Asp^{255} and Asp^{299} on PEDF are essential residues for collagen binding, and Asp^{257} is also important in the interaction.

A similar binding assay for Lys→Ala and Arg→Ala mutants also resulted in the identification of residues critical for the PEDF−heparin interaction (Figure 6B). Although this series of mutants showed collagen-binding activity similar to the wild-type PEDF (Figure 6B, top panel), a marked reduction in heparin-binding activity was observed for K146A and R148A (Figure 6B, middle panel). In addition, the R145A mutant also showed a moderate reduction in heparin-binding activity (Figure 6B, middle panel). Therefore, we conclude that three basic amino acid residues (Arg^{145}, Lys^{146}, and Arg^{148}) largely contribute to the PEDF−heparin interaction. The CD spectra of these mutant proteins with reduced ECM-binding activity indicated that the mutagenesis did not perturb the secondary structure of rPEDF, and their reduced affinity for ECM components is not due to an abnormal conformation (Figure 7).

DISCUSSION

Considerable numbers of secreted proteins such as growth factors and cytokines are known to interact with ECM. To date, ECM-binding has been shown for basic fibroblast growth factor (*28*, *29*), transforming growth factor β (*30*), interleukin-2 (*31*), and oncostatin M (*32*), etc., and the ECM-binding is thought to be important for the expression of their biological activities.

PEDF is a secreted protein found in the interphotoreceptor matrix that interacts with collagens (*17*) and GAGs (*15*, *16*). Several other serpins such as HSP47 and maspin also bind to collagens. HSP47 is a procollagen-specific molecular chaperone that resides in the endoplasmic reticulum (*33*, *34*). HSP47 binds to various types of collagen tested to date, whereas PEDF binds to collagen I and III but not II and IV (*17*). Recently, it was demonstrated that Xaa−Arg−Gly triplets in the collagen triple helix are dominant binding sites for HSP47 (*21*). In the present study, we demonstrated that PEDF interacts with collagen I mainly by ionic interaction unlike HSP47 (Figure 2), and PEDF recognizes both Lys and Arg residues on collagen I (Figure 4). PEDF does not bind to the collagen model peptide containing a Xaa−Arg−Gly sequence (data not shown). Taking this information into consideration, PEDF seems to recognize amino acid sequences on collagen more strictly than HSP47. These differences in the collagen-binding profiles of PEDF and HSP47 suggest that these serpins might have independently acquired their collagen-binding activity during evolution.

Recently, Blaque and Worrall demonstrated that maspin, another anti-angiogenic serpin, binds to collagen I and III (*20*). They proposed that collagen binding might be involved in an anti-angiogenic activity of serpins, based on the similarity in the collagen-binding property between PEDF and maspin. They also reported that the maspin-binding sequence in collagen α2 (I) is located within Arg^{708}−Glu^{923} (accession no. Z74616) (*20*). It is still unknown whether PEDF also recognizes the sequence or not. Determination of PEDF-binding sites on collagen and a comparison between those of PEDF and maspin will be of help in understanding the molecular function of such anti-angiogenic serpins in the future.

By means of chemical modification, we demonstrated that negatively charged residues of PEDF were involved in collagen binding (Figure 5A). On the basis of the crystal structure of human PEDF, the molecule has distinct asymmetric charge distribution (ref *22*, Figure 8). Taking this unique charge distribution into consideration, we replaced Asp and Glu residues on the acidic surface with Asn and Gln, respectively (Figure 8, left). Using these mutants, we revealed that Asp^{299} in helix H, Asp^{255} in sheet 1B, and Asp^{257} are important in the interaction between PEDF and collagens (Figures 6 and 8). The collagen-binding region of maspin has been reported to be located between amino acids 84 and 112 (*20*). This region consists of the secondary structure, sheet 2A and helix E, according to the modeled structure of maspin (*35*). Therefore, the spatial localization of the collagen-binding site on PEDF is different from that of maspin, although similarity in collagen-binding properties of these anti-angiogenic serpins is reported (*20*).

Previously, it has been proposed that the heparin-binding site is localized at the basic surface of PEDF (*15*, *22*). To understand PEDF−heparin interaction in depth, Lys and Arg residues composing the basic surface were substituted with Ala, and the heparin binding of these mutants was examined. We consequently found that Arg^{145}, Lys^{146}, and Arg^{148} on PEDF are critical to heparin binding. To date, several serpins are known to interact with heparin. Heparin-binding sites of antithrombin (*36*), heparin cofactor II (*37*), proteinase nexin I (*38*), and plasminogen activator inhibitor I (*39*) have been localized on helix D. Protein C inhibitor (*40*) and kallistatin (*41*) have been reported to bind to heparin using helix H and the region between helix H and sheet 2C, respectively. The basic amino acid residues on PEDF involved in heparin binding, identified in this study, are located in the loop region between sheet 2A and helix E (Figure 8, right). To our

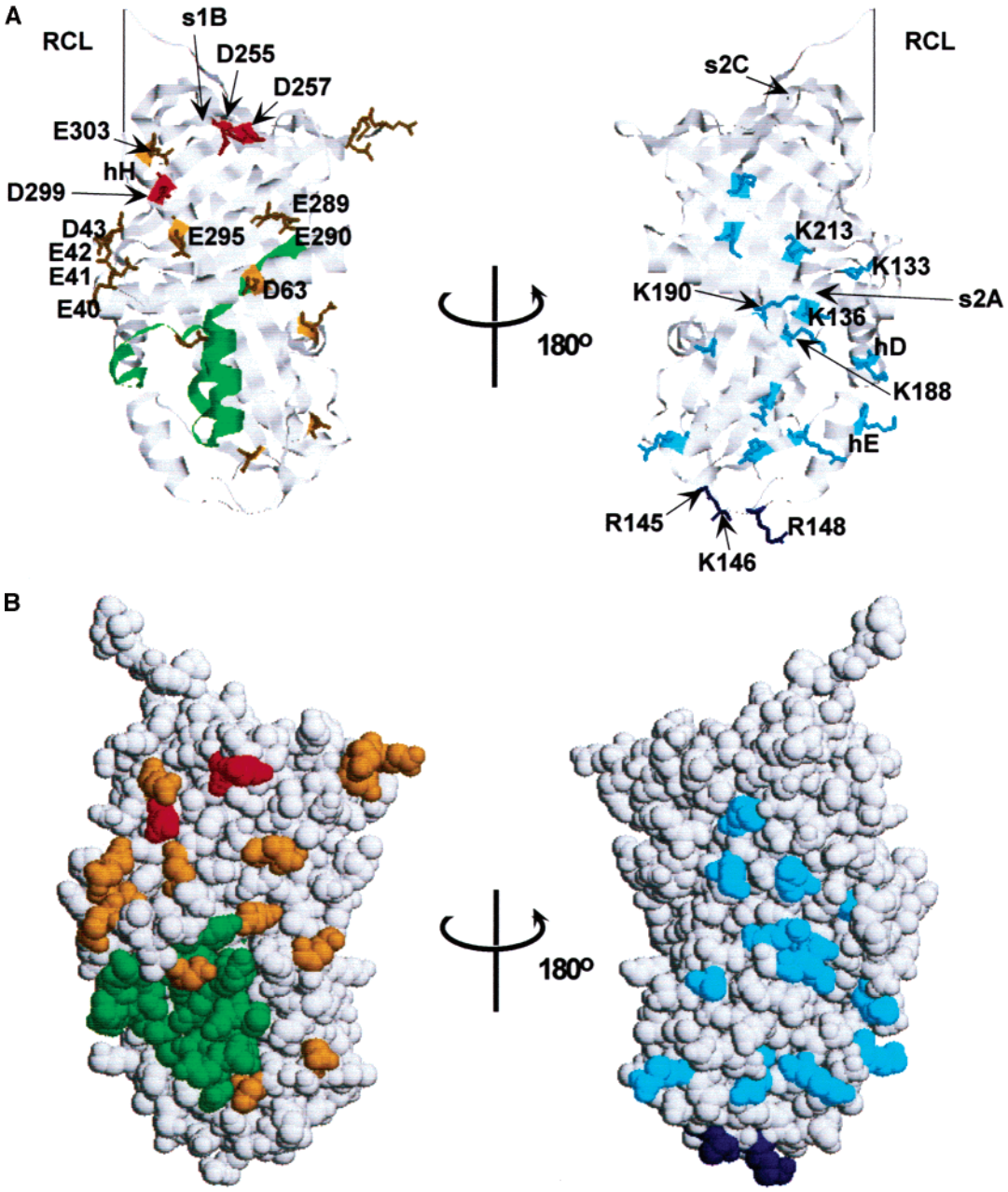

FIGURE 8: Spatial distribution of the binding sites for collagen I and heparin on PEDF. The collagen-binding site localizes to the side opposite the heparin-binding site. Ribbons diagrams (A) and spacefilling representation (B) of PEDF (PDB accession code: 1IMV, ref *22*) are illustrated. (Left) The acidic amino residues mutated in this study are in orange, and Asp residues critical for collagen binding are in red. The putative region involved in neurotrophic activities of PEDF is shown in green. (Right) Lys and Arg residues mutated in this study are in cyan, and amino acid residues critical for heparin binding are in dark blue. The view is from the opposite side illustrated in the left panels. RCL is labeled for the reactive center loop. This figure was made with RasMol.

knowledge, this region is a novel-type heparin-binding site in serpins.

Mapping of the amino acid residues critical to the ECM-binding on the X-ray crystallographic structure demonstrated that the collagen-binding site does not overlap the heparin-binding site on PEDF (Figure 8). This conclusion is consistent with that made by Meyer et al. in a recent report (*42*). They proposed that Glu^{40}, Glu^{41}, Glu^{42}, Asp^{43}, Asp^{63}, Glu^{289}, Glu^{290}, Glu^{295}, and Glu^{303} are available to interact with collagen based on a similar chemical modification study. However, our site-directed mutagenesis study has clearly demonstrated that Asp^{255}, Asp^{257}, and Asp^{299} are critical to collagen recognition by PEDF, and other acidic residues are not so important. We speculated that the spatial distribution of negatively charged Asp^{255}, Asp^{257}, and Asp^{299} side chains might be suitable to form specific salt bridges with Lys and Arg side chains in the PEDF-binding site on collagen. The site-directed mutagenesis study has also demonstrated that Arg^{145}, Lys^{146}, and Arg^{148} mainly contribute to the specific interaction with heparin, and other basic residues are not important. It should be noted that the identified heparin-binding sequence, Arg^{145}−Lys−Leu−Arg^{148}, matches to heparin-binding consensus, −X−B−B−X−B−X (*43*). We think that the positive charges of Lys^{133}, Lys^{136}, Lys^{188}, Lys^{190}, Arg^{193}, and Lys^{213} proposed by Meyer et al. (*42*) as the putative heparin-binding sites may contribute to bulk electrostatic interactions with positive charges in the heparin rather than to specific recognition of heparin.

The spatial relationship of collagen and heparin binding sites is of interest and allows us to speculate on the possible mechanisms of PEDF action. First, PEDF might act in ECM as a linking protein. PEDF could link collagens with GAGs by simultaneously binding to both collagens and GAGs. It is worth noting that interaction between GAGs on the endothelial cell surface and collagen I could be important in angiogenesis (*44*). Second, PEDF actions against different target cells could be regulated by its binding to different ECM components. A previous report described that the region involved in the neurotrophic activity of PEDF is localized in the N-terminal region spanning Val^{78}−Thr^{121} and that the 44-mer peptide corresponding to this region has the ability to bind to the putative PEDF receptor (*13*). Moreover, this region is oriented toward the opposite side of the heparin-binding site (*15*, *22*). Thus, it was expected that receptor binding of PEDF might be achieved by the interaction with GAGs around the surface of target cells (*15*). Interestingly, the neurotrophic region is localized to the acidic surface of PEDF where the collagen-binding site is located. This spatial relationship implies that collagen binding could negatively modify the neurotrophic activity by shielding the neurotrophic region. It is tempting to speculate that the unique ECM binding properties of PEDF are involved in several biological activities such as neuronal survival, cell differentiation, and anti-angiogenesis by switching the surfaces for binding to different ECM components. However, the relationship between the biological activities of PEDF and its ECM binding properties is unknown. PEDF point mutants obtained in this study, which lost the ability to bind to ECM components, will be powerful tools to investigate the relationship between the functions and the ECM binding of PEDF.

SUPPORTING INFORMATION AVAILABLE

Tables of mutagenic primers. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Taipale, J., and Koseki-Oja, J. (1997) *FASEB J. 11*, 51−59.
2. Tanaka, Y., Kimata, K., Adams, D. H., and Eto, S. (1998) *Proc. Assoc. Am. Physicians 110*, 118−125.
3. Steele, F. R., Chader, G. J., Johnson, L. V., and Tombran-Tink, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1526−1530.
4. Becerra, S. P., Sagasti, A., Spinella, P., and Notario, V. (1995) *J. Biol. Chem. 270*, 25992−25999.
5. Tombran-Tink, J., Chader, G. J., and Johnson, L. V. (1991) *Exp. Eye Res. 53*, 411−414.
6. Taniwaki, T., Becerra, S. P., Chader, G. J., and Schwartz, J. P. (1995) *J. Neurochem. 64*, 2509−2517.
7. Taniwaki, T., Hirashima, N., Becerra, S. P., Chader, G. J., Etcheberrigaray, R., and Schwartz, J. P. (1997) *J. Neurochem. 68*, 26−32.
8. Bilak, M. M., Corse, A. M., Bilak, S. R., Lehar, M., Tombran-Tink, J., and Kuncl, R. W. (1999) *J. Neuropathol. Exp. Neurol. 58*, 719−728.
9. Houenou, L. J., D'Costa, A. P., Li, L., Trugeon, V. L., Enyadike, C., Alberdi, E., and Becerra, S. P. (1999) *J. Comp. Neurol. 412*, 506−514.
10. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H.-J., Benedict, W., and Bouck, N. P. (1999) *Science 285*, 245−248.
11. Stellmach, V., Crawford, S. E., Zhou, W., and Bouck, N. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 2593−2597.
12. Volpert, O. V., Zaichuk, T., Zhou, W., Reiher, F., Ferguson, T. A., Stuari, P. M., Amin, M., and Bouck, N. P. (2002) *Nat. Med. 8*, 349−357.
13. Alberdi, E., Aymerich, M. S., and Becerra, S. P. (1999) *J. Biol. Chem. 274*, 31605−31612.
14. Aymerich, M. S., Alberdi, E., Martinez, A., and Becerra, S. P. (2001) *Invest. Opthalmol. Vis. Sci. 42*, 3287−3293.
15. Alberdi, E., Hyde, C. C., and Becerra, S. P. (1998) *Biochemistry 37*, 10643−10652.
16. Stratikos, E., Alberdi, E., Gettins, P. G. W., and Becerra, S. P. (1996) *Protein Sci. 5*, 2575−2582.
17. Kozaki, K., Miyaishi, O., Koiwai, O., Yasui, Y., Kashiwai, A., Nishikawa, Y., Shimizu, S., and Saga, S. (1998) *J. Biol. Chem. 273*, 15125−15130.
18. Chader, G. J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 2122−2124.
19. Zheng, M., Volpert, O., Shi, Y. H., and Bouck, N. P. (2000) *Nat. Med. 6*, 196−199.
20. Blacque, O. E., and Worrall, D. M. (2002) *J. Biol. Chem. 277*, 10783−10788.
21. Koide, T., Takahara, Y., Asada, S., and Nagata, K. (2002) *J. Biol. Chem. 277*, 6178−6182.
22. Simonovic, M., Gettins, P. G. W., and Volz, K. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 11131−11135.
23. Higuchi, R., Kurummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res. 16*, 7351−7367.
24. Koide, T., Asada, S., and Nagata, K. (1999) *J. Biol. Chem. 274*, 34523−34526.
25. Asada, S., Koide, T., Yasui, H., and Nagata, K. (1999) *Cell Struct. Funct. 24*, 187−196.
26. Natsume, T., Koide, T., Yokota, S., Hirayoshi, K., and Nagata, K. (1994) *J. Biol. Chem. 269*, 31224−31228.
27. Saga, S., Nagata, K., Chen, W. T., and Yamada, K. M. (1987) *J. Cell. Biol. 105*, 517−527.
28. Powers, C. J., McLeskey, S. W., and Wellstein, A. (2000) *Endocr. Relat. Cancer 7*, 165−197.
29. Pellegrini, L., Burke, D. F., von Derft, F., Mulloy, B., and Blundell, T. L. (2000) *Nature 407*, 1029−1034.
30. Koli, K., Saharinen, J., Hyytäiinen, M., Penttinen, C., and Keski-Oja, J. (2001) *Microsc. Res. Tech. 52*, 354−362.
31. Somasundararn, R., Ruehl, M., Tiling, N., Ackermann, R., Schmid, M., Riecken, E. O., and Schuppan, D. (2000) *J. Biol. Chem. 275*, 38170−38175.
32. Somasundaram, R., Ruehl, M., Schaefer, B., Schmid, M., Ackermann, R., Riecken, E. O., Zeitz, M., and Schuppan, D. (2002) *J. Biol. Chem. 277*, 3242−3246.
33. Nagata, K. (1996) *Trends Biochem. Sci. 21*, 22−26.
34. Hendershot, L. M., and Bulleid, N. J. (2000) *Curr. Biol. 10*, 912−915.
35. Fizpatrick, P. A., Wong, D. T., Barr, P. J., and Pemberton, P. A. (1996) *Protein Eng. 9*, 585−589.
36. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14683−14688.
37. Whinna, H. C., Blinder, M. A., Szewczyk, M., Tollefsen, D. M., and Church, F. C. (1991) *J. Biol. Chem. 266*, 8129−8135.
38. Stone, S. R., Brown-Luedi, M. L., Rovelli, G., Guidolin, A., McGlynn, E., and Monard, D. (1994) *Biochemistry 33*, 7731−7735.
39. Ehrlich, H. J., Gebbink, R. K., Keijer, J., and Pannekoek (1992) *J. Biol. Chem. 267*, 11606−11611.
40. Shirk, R. A., Elisen, M. G. L. M., Meijers, J. C. M., and Church, F. C. (1994) *J. Biol. Chem. 269*, 28690−28695.
41. Chen, V. C., Chao, L., Pimenta, D. C., Bledsoe, G., Juliano, L., and Chao, J. (2001) *J. Biol. Chem. 276*, 1276−1284.
42. Meyer, C., Notari, L., and Becerra, S. P. (2002) *J. Biol. Chem. 277*, 45400−45407.
43. Cardin, A. D., and Weintraub, H. J. R. (1989) *Arteriosclerosis 9*, 21−32.
44. Sweeney, S. M., Guy, C. A., Fields, G. B., and San Antonio, J. D. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 7275−7280.

BI0206558