

Characterization of Molecular Interactions between Eosinophil Cationic Protein and Heparin*

Received for publication, May 8, 2008 Published, JBC Papers in Press, June 30, 2008, DOI 10.1074/jbc.M803516200

Tan-chi Fan[‡], Shun-lung Fang[‡], Chi-shin Hwang^{‡§}, Chih-yen Hsu[‡], Xin-an Lu[¶], Shang-cheng Hung^{¶||}, Shu-Chuan Lin[‡], and Margaret Dah-Tsyr Chang^{‡¶1}

From the [‡]Institute of Molecular and Cellular Biology and Department of Life Science, National Tsing Hua University, 101, Section 2, Kuang Fu Road, Hsinchu 30013, the [§]Department of Neurology, Taipei City Hospital, Zhong Xiao Branch, Taipei 10341, the [¶]Department of Chemistry, National Tsing Hua University, Hsinchu 30013, and the ^{||}Genomics Research Center, Academia Sinica, Taipei 11529, Taiwan

Eosinophil cationic protein (ECP) is currently used as a biomarker for airway inflammation. It is a heparin-binding ribonuclease released by activated eosinophils. Its cytotoxicity toward cancer cell lines is blocked by heparin. The objective of this study was to locate the heparin binding site of ECP by site-directed mutagenesis and construction of a synthetic peptide derived from this region. Synthetic heparin with ≥ 5 monosaccharide units showed strong inhibition of ECP binding to the cell surface. Analysis of ECP mt1 (R34A/W35A/R36A/K38A) showed that these charged and aromatic residues were involved in ECP binding to heparin and the cell surface. A potential binding motif is located in the loop L3 region between helix $\alpha 2$ and strand $\beta 1$, outside the RNA binding domain. The synthetic peptide derived from the loop L3 region displayed strong pentasaccharide binding affinity and blocked ECP binding to cells. In addition, ECP mt1 showed reduced cytotoxicity. Thus, the tight interaction between ECP and heparin acts as the primary step for ECP endocytosis. These results provide new insights into the structure and function of ECP for anti-asthma therapy.

Eosinophil cationic protein (ECP),² a member of the ribonuclease A (RNase A) superfamily, is found in the specific granules of eosinophilic leukocytes. It is a single polypeptide with a molecular mass ranging from 16 to 21.4 kDa due to varying degrees of glycosylation. It shows a 67% amino acid sequence identity with eosinophil-derived neurotoxin (EDN), another

eosinophil-secreted RNase. Although ECP shares the overall three-dimensional structure of RNase A, it has relatively lower RNase activity (1). ECP released by activated eosinophils contributes to the toxicity against helminth parasites, bacteria, and single strand RNA viruses (2–4). Together with other proteins secreted from eosinophils, ECP is thought to cause damage to epithelial cells, a common feature of airway inflammation in asthma (5).

The mechanism underlying the cytotoxic property of ECP is unclear. It has been hypothesized that ECP cytotoxicity is due to destabilization of lipid membranes of target cells (6), and the degree of cytotoxicity is dependent on the cellular concentration (7). The binding of ECP to target cells has been attributed to its high arginine content (estimated pI = 10.8), which facilitates the interaction between ECP and negatively charged molecules on the cell surface (7, 8). Recently, we found that binding and endocytosis of ECP into bronchial epithelial cells were greatly dependent on the cell surface glycosaminoglycan, specifically heparan sulfate proteoglycans (HSPG) (9). The cytotoxicity of ECP was severely reduced toward cell lines with heparan sulfate (HS) deficiency.

Heparin and HS are complex polysaccharides composed of alternating units of hexuronic acid and glucosamine. The uronic acid residues of heparin typically consist of 90% L-idopyranosyluronic acid and 10% D-glucopyranosyluronic acid (10). The N position of glucosamine may be substituted with an acetyl or sulfate group. The 3-O and 6-O positions of glucosamine and the 2-O of uronic acid may be sulfated. Through the combination of different negatively charged moieties, heparin and HS have been demonstrated to bind a variety of proteins with diverse functions, including growth factors, thrombin, chemokines, and viral proteins. The HS chains contain domains with a high level of sulfation and epimerization (S-domains), regions with mixed N-acetylation and N-sulfation (NA/S-domains), and unmodified domains that are mostly N-acetylated and contain little sulfate (11). Because HS chains contain heparin regions, heparin and its mimetics can be used to study interactions between proteins and polysaccharides.

The structure of ECP has been determined and refined to a resolution up to 1.75 Å, displaying a folding topology that involves three α helices and five β strands (12). The most interesting feature is as many as 19 surface-oriented arginine residues, conferring a strong basic character to ECP. However, the heparin binding site in ECP has not been identified. Heparin

* This work was supported by National Science Council, China (Grants NSC97-3112-B-007-006, NSC97-2752-B-007-001-PAE, NSC97-2752-B-007-003-PAE, NSC96-2627-M-007-002, NSC95-2113-M-007-028-MY3, NSC96-2752-B-007-002-PAE), by the Chang-Gung Memorial Hospital-National Tsing Hua University (Joint Research Grant CGTH96-T12), and by the Veterans General Hospitals University System of Taiwan Joint Research Program (Grant VGHUST97-G2-4). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed. Tel.: 886-3-574-2767; Fax: 886-3-571-5934; E-mail address: dtchang@life.nthu.edu.tw.

² The abbreviations used are: ECP, eosinophil cationic protein; AMAC, 2-aminoacridone; EDN, eosinophil-derived neurotoxin; FACE, fluorescence-assisted carbohydrate electrophoresis; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; ITFE, intrinsic tryptophan fluorescence emission; LMWH, low molecular weight heparin; MBP, maltose-binding protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HRP, horseradish peroxidase.

binding domains within proteins usually contain a high proportion of positively charged residues, which bind to the acidic groups of heparin through electrostatic interactions. It has been proposed that the three-dimensional structure of the HS chain is critical for protein binding (13). However, not much is known about the three-dimensional structure of HS. After examining a series of heparin-binding protein sequences, Cardin and Weintraub (14) proposed that the pattern XBBBXXBX or XBBXXBX (where *X* represents hydrophobic or uncharged amino acids, and *B* represents basic amino acids) is responsible for HS binding to other proteins.

In the present work, a linear heparin binding site on ECP and the shortest ECP-binding heparin oligosaccharide unit have been identified. Loop L3 of ECP may mediate the interaction between ECP and cell surface HSPG, contributing to ECP cytotoxicity. Furthermore, the dissociation constants between oligosaccharides and ECP were determined by tryptophan emission titration.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse anti-maltose-binding protein (MBP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RNase A was purchased from Promega (Madison, WI). Chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Recombinant Protein Purification—Recombinant human ECP containing a C-terminal His₆ tag was expressed in *Escherichia coli* BL21 codon plus (DE3) (Novagen, Madison, WI), purified by chromatography, and refolded as previously described (1). Amino acid residues Arg-34, Trp-35, Arg-36, and Lys-38 of ECP were simultaneously substituted to alanine using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), and the resultant mutant was named ECP mt1. The primers used were as follows: mt1 forward, 5'-TATGCAGCGGCTTGCGCAAACCAAAT-3'; and mt1 reverse, 5'-TTTGCGCAAGCCGCTGCATAATTGTTA-3'. *E. coli* BL21 codon plus (DE3) cells were used to transform various plasmids. MBP-ECP was purified using amylose affinity chromatography (1).

Cells and Cell Culture—Beas-2B, a human bronchial epithelial cell line, was cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen).

Fluorescence-assisted Carbohydrate Electrophoresis—Synthetic heparin oligosaccharide from the degree of polymerization (dp) 3–9 (Fig. 1, *M_r* 926–2642) (16), low molecular weight heparin (LMWH, Sigma-Aldrich, average *M_r* 3,000), and PI-88 (kindly provided by Progen Inc., Australia) were labeled with 2-aminoacridone (AMAC) as described previously (17). AMAC-oligosaccharide and proteins were incubated at room temperature for 15 min, loaded onto 1% gels, and electrophoresed as described (18).

Cell ELISA—The ability of MBP-ECP to bind cells in the presence of serial dilutions of oligosaccharides or peptides was determined as described (9). Briefly, confluent monolayers of Beas-2B cells in 96-well plates were pretreated with various concentrations of oligosaccharides or peptides in serum-free RPMI 1640 medium at 4 °C for 30 min before incubation with 5 μg/ml MBP-ECP at 4 °C for 1 h. The cells were then washed

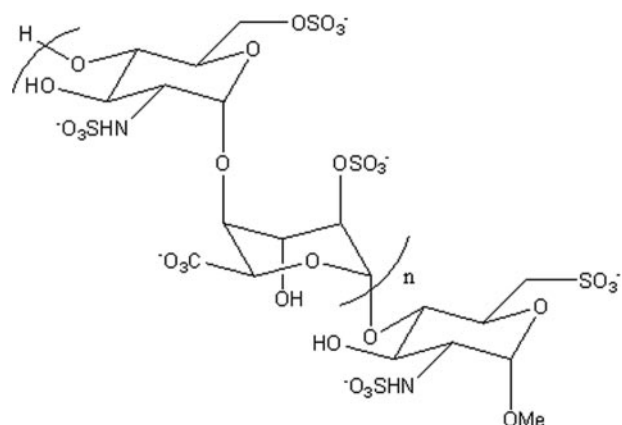


FIGURE 1. The structure of synthetic heparin oligosaccharides used in this study. *n* = 1–4.

with ice-cold PBS and fixed with 2% paraformaldehyde at room temperature for 15 min prior to blocking with 2% bovine serum albumin/PBS at room temperature for 90 min. The level of bound MBP-ECP was quantified by ELISA analysis. MBP-ECP was detected using mouse monoclonal anti-MBP and goat anti-mouse HRP-conjugated secondary antibody, followed by the enhanced chemiluminescence detection system. The amount of MBP-ECP bound to cells without oligosaccharide or peptide treatment was set to 100%.

Intrinsic Tryptophan Fluorescence Emission—Binding of ECP to heparin was monitored by changes in ITFE. ECP (200 nM) in PBS at 25 °C was titrated with small aliquots of a high concentration of heparin oligosaccharides with minimal dilution (<2%). Protein fluorescence measurements were recorded 2 min after each addition on a Hitachi 8000 spectrofluorometer at emission wavelength of 340 nm using an excitation wavelength of 280 nm. ΔF , the relative fluorescence change, equals $F_0 - F_{\text{obs}}$, where F_0 and F_{obs} represent the initial and observed fluorescence values, respectively. Binding constants were estimated from the titration data using a nonlinear least-squares computer fit to the equation based on 1:1 binding stoichiometry (19),

$$\Delta F = \Delta F_{\text{max}} \times ([P] + [H] + K_d - (([P] + [H] + K_d)^2 - 4 \times [P] \times [H])^{1/2}) / (2 \times [P]) \quad (\text{Eq. 1})$$

where ΔF_{max} is the maximum relative fluorescence change, P is the total concentration of ECP, H is the concentration of heparin, and K_d is the dissociation constant for the ECP-heparin interaction.

CD Spectroscopy—CD spectra were recorded using an Aviv model 202 CD Spectrometer equipped with a 450-watt xenon arc lamp. Far-UV spectra were recorded at 25 °C from 200 to 250 nm using a 0.1-cm cuvette containing 10 μM protein in PBS. The CD spectra were recorded at 1.5-min intervals with a bandwidth of 1 nm. Each spectrum is an average of three consecutive scans and was corrected by subtracting the buffer spectrum.

Cell Growth Assay—The effect of ECP on the cell growth was determined by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (US Biological, Marblehead, MA). Cells were plated in a 96-well plate (5000 cells/well) and incubated at 37 °C overnight. Each sample

was incubated with the indicated concentration (1–100 μM) of ECP. Forty-eight hours after treatment with ECP, MTT was added, and cell growth was monitored at A_{570} to measure the mitochondrial-dependent formation of a colored product.

Synthetic Peptides—Human ECP peptide NYRWCKN-QNK-biotin (C1), EDN peptide NYQRRCKNQNK-biotin (D1), RNase1 peptide MTQGRCKPVNK-biotin (R1), and HIV-TAT peptide YGRKKRRQRRRK-biotin (Tat) were purchased from Genemed Synthesis (South San Francisco, CA).

Immunohistochemical Staining—Adult specific-pathogen-free Sprague-Dawley rats (Narl:SD), with body weight of 200–300 g, were purchased and maintained at the National Laboratory Animal Center in Taiwan and heparin (FRAGMIN®, average M_r 6,000 and was 5,000 IU/0.2 ml). The rats were separated into three groups. In group 1, each rat was injected with 5 nmol of ECP through the tail vein. In group 2, each rat was co-injected with heparin (20 IU/100 g of body weight) and 5 nmol of ECP. In group 3, each rat was injected with 5 nmol of MBP as the negative control. All animals were sacrificed using CO_2 narcosis 1 h after the injection of these agents. The lung and trachea of these rats were taken and immediately fixed with 10% neutral-buffered formaldehyde. The tissue samples were processed by routine methods to prepare paraffin wax-embedded block. These blocks were then sectioned into 6- μm slices. All tissue sections were examined using the Super Sensitive Non-Biotin HRP Detection System (BioGenex Laboratories, San Ramon, CA) as described (20). Briefly, the mouse anti-ECP or anti-MBP monoclonal antibody was used as the primary antibody. Antigen unmasking was performed by immersion of sections in 5% Trilogy (Cell Marque, Rocklin, CA) antigen unmasking solution in Milli-Q water and boiled at 121 °C. Endogenous peroxidase activity was quenched with hydrogen peroxide (3%) in methanol. These sections were then incubated in Power Block solution, and mouse anti-ECP or anti-MBP at 1/200 dilution was applied and left for 24 h. The sections were incubated with Super Enhancer reagent, followed by Polymer-HRP reagent, and then incubated with 3-amino-9-ethylcarbazole chromogen solution. The sections were finally counterstained with Mayer's hematoxylin and mounted with Super Mount permanent aqueous mounting media prior to examination with a light microscope (Axioplan, Zeiss, Germany).

RESULTS

Oligosaccharide Length Dependence of ECP-Heparin Interaction—Previously, we demonstrated that binding and endocytosis of ECP are HSPG-dependent (9). An early study demonstrated ECP purification using heparin-Sepharose chromatography (21). Thus, the minimal heparin length (or disaccharide unit) required to interact with ECP was investigated by FACE. Initially, ECP was co-incubated with AMAC-labeled LMWH at various molar ratios, and the binding products were analyzed by gel electrophoresis. The decrease in the free LMWH signal was monitored as ECP concentration was increased (Fig. 2A). Subsequently, the dependence of ECP binding on heparin size was examined in the presence of synthetic heparin oligosaccharides dp3 to dp9 using LMWH as a positive control. It was apparent that dp5 served as the shortest heparin fragment that retained the ability to bind ECP (Fig. 2B). Fur-

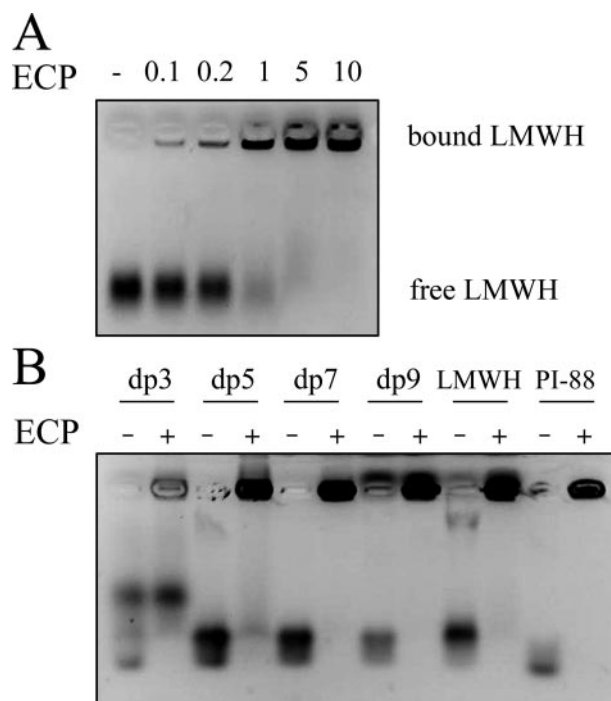


FIGURE 2. FACE analysis to evaluate binding of ECP to various sugar-containing substrates. A, AMAC-labeled LMWH (0.03 nmol) was incubated with or without ECP in PBS for 10 min at 25 °C, and the binding reaction products were separated on a 1% agarose gel. The numbers above the gel indicate the molar ratio of ECP to LMWH. B, representative oligosaccharide binding pattern of ECP. AMAC-labeled heparin dp3 (10.8 nmol), dp5 (3.3 nmol), dp7 (1.4 nmol), dp9 (1.1 nmol), LMWH (0.03 nmol), and PI-88 (0.03 nmol) were incubated with or without 10-fold molar excess of ECP, and the binding reaction products were separated on a 1% agarose gel.

thermore, we tested a potent heparanase inhibitor (PI-88), undergoing clinical trials for its anti-angiogenic and anti-metastatic effects (22, 23), that contains a mixture of highly sulfated mannose-containing di- to hexasaccharides (24, 25). Interestingly, this heparin mimetic could also bind ECP (Fig. 2B).

Competitive Inhibition of ECP Binding to Cells by Synthetic Oligosaccharides—Using cell ELISA, synthetic heparins (dp3–dp9) were tested for their ability to interfere with ECP binding to Beas-2B cells. Beas-2B cells were preincubated with oligosaccharides, and bound ECP was detected essentially as described (9). The degree of inhibition increased with increasing oligosaccharide length (Fig. 3). 50 $\mu\text{g}/\text{ml}$ heparin dp5 inhibited 50% of ECP binding to cells, and the same amount of dp7 and dp9 was capable of inhibiting over 70 and 80% of ECP binding, respectively. These data revealed that pentasaccharide was the minimal length sufficient to interfere with ECP binding to Beas-2B cells. In addition, the concentration dependence of PI-88 against cellular binding of ECP was similar to that of dp7 (Fig. 3).

Identification of Heparin-binding Sequence in ECP—A consensus sequence of the heparin binding site (e.g. XBBXB or XBBBXXB) has been found in many glycosaminoglycan-binding proteins or peptides (3, 14). Inspection of the sequences of human RNase A family members revealed a surface loop L3 region, $^{34}\text{QRRCKN}^{39}$, in EDN that exactly matched the XBBXB motif (Fig. 4A), but no consensus heparin-binding motif was found in ECP. Therefore, we speculated that residues 34–39 ($^{34}\text{RWRCCKN}^{39}$) in ECP that correspond to the consen-

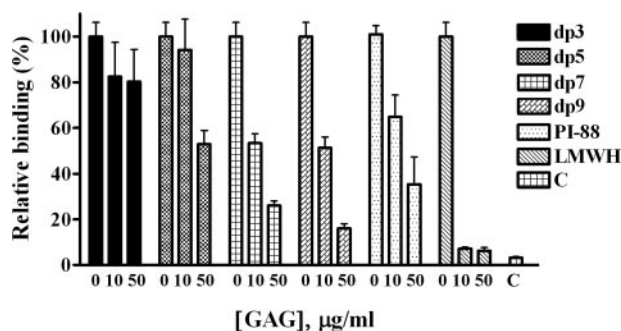


FIGURE 3. Heparin oligosaccharides inhibit ECP binding to cells. Beas-2B cells were preincubated with heparin of different sizes in RPMI 1640 medium for 30 min at 4 °C before incubation with MBP-ECP for an additional 1 h. After treatment, the cells were washed with PBS and fixed with paraformaldehyde. The level of bound MBP-ECP was assessed by ELISA. The amount of MBP-ECP bound to cells without glycosaminoglycan treatment was set to 100%. C, control (cells were incubated with MBP). The data shown are the means of triplicate experiments.

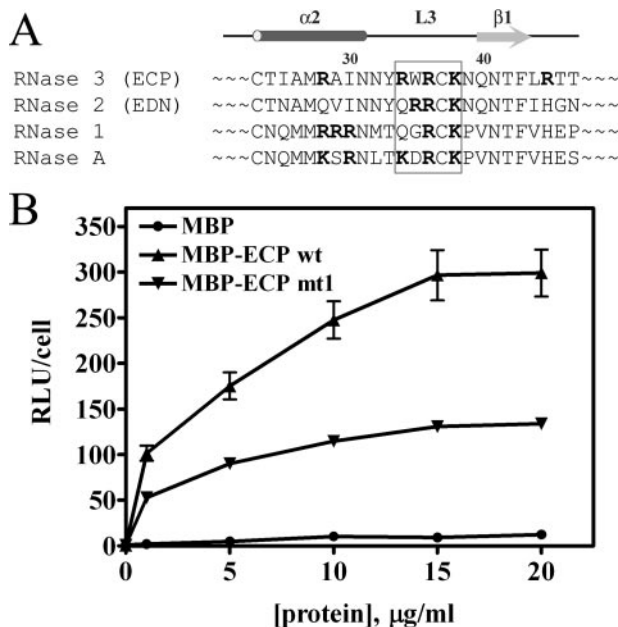


FIGURE 4. Identification of the heparin binding site in ECP. A, alignment human and bovine RNases. The sequences between $\alpha 2$ and $\beta 1$ of the RNases are aligned. Regions in other RNases that are highly similar to the putative heparin binding site of ECP are boxed. Positively charged amino acids are indicated in bold. B, Beas-2B cells were incubated with MBP-ECP for 1 h at 4 °C. The amount of MBP-ECP bound to cells was assessed as for Fig. 3.

sus motif in EDN might also bind heparin. To determine whether this region contributes to cellular binding, cell ELISA analysis was conducted using MBP-ECP and MBP-ECP mt1 containing the mutations R34A/W35A/R36A/K38A. This mutant had only 50% of the cell-binding activity, indicating the importance of the $^{34}\text{RWRCKN}^{39}$ motif in ECP for cellular HS binding (Fig. 4B).

Characterization of Association between ECP and Heparin Oligosaccharides—The binding affinities of wild-type and mutant ECP for heparin oligosaccharides were subsequently monitored by intrinsic tryptophan fluorescence titration (27). The change in tryptophan fluorescence revealed that wild-type ECP bound to dp5 with high affinity, and the corresponding K_d was 139.6 nM (Fig. 5). As expected, a 4- to 5-fold increase in K_d to 568.1 nM was observed for ECP mt1 (R34A/W35A/R36A/

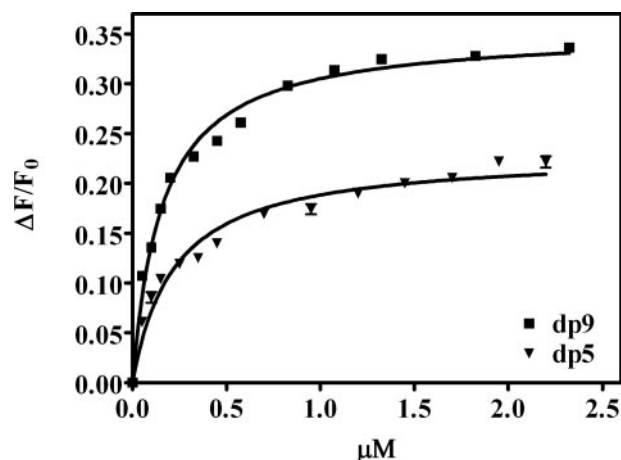


FIGURE 5. ITFE profiles of ECP wt and ECP mt1 with dp5. Tryptophan titration emission spectra of 0.2 mM ECP wt or ECP mt1 bound with dp5. The emission spectra at 340 nm were recorded. The resulting isotherms were fitted by nonlinear regression least-squares computer fit using the Kaleida-Graph Synergy Software. ΔF , the relative fluorescence change, equals $F_0 - F_{\text{obs}}$, where F_0 and F_{obs} represent the initial and observed fluorescence values, respectively.

TABLE 1

Dissociation constant (K_d) determination for wild-type ECP and ECP mt1 with heparin oligosaccharides

K_d was measured in PBS at 25 °C. The change in intrinsic tryptophan fluorescence was fit by the equation 1 to obtain K_d .

Oligosaccharide	K_d	
	ECP wt	ECP mt1
	nM	
dp9	62.6	400.3
dp5	139.6	568.1

K38A) (Table 1), indicating decreased heparin-binding activity for mutant ECP lacking the key heparin-binding motif in the L3 region.

CD spectroscopy to compare the conformations of wild-type ECP and ECP mt1 showed that the secondary structure of ECP mt1 was very similar to that of wild-type ECP. These results strongly indicated that the decrease in heparin binding affinity resulted from a loss of the specific recognition sequence motif, but not from a conformational change in ECP mt1.

Competitive Inhibition of ECP Binding to Cells by Synthetic Peptides—To investigate whether the RWRCK motif was directly responsible for heparin binding, cell ELISA was conducted using a synthetic peptide, C1 (NYRWRCKNQNK), along with a positive control peptide derived from HIV-TAT, YGRKKRRQRRRK (28, 29). The dose-dependent competition of these peptides is shown in Fig. 6A. Binding of MBP-ECP to cell-surface HS was significantly reduced in the presence of both C1 and TAT peptides. Therefore, synthetic peptides with heparin-binding activity may compete with ECP for cellular binding. Furthermore, the cell surface-binding ability of peptides, including D1 (NYQRRCKNQNK) derived from EDN and R1 (MTQGRCKPVNK) derived from RNase1 was tested using cell ELISA assay. As expected, similar to the TAT peptide, C1 and D1 peptides bound to the cell surface, whereas R1 peptide was devoid of such function (Fig. 6B).

Interaction of Synthetic Peptides with LMWH—The ability of C1 to directly interact with LMWH was further investigated by

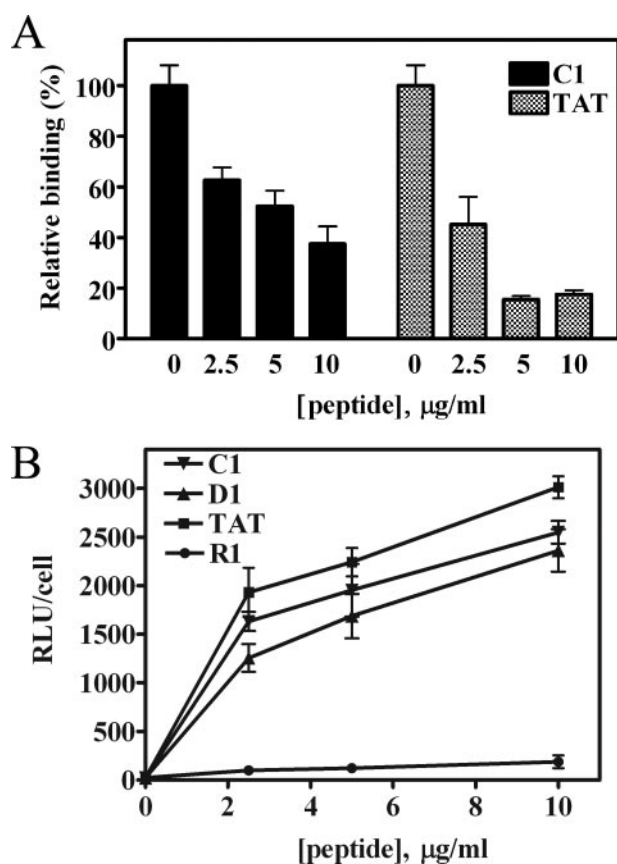


FIGURE 6. Synthetic peptides inhibit ECP binding to cells. A, Beas-2B cells were preincubated with peptides in RPMI 1640 medium for 30 min at 4 °C before incubation with MBP-ECP for an additional 1 h. After treatment, the cells were washed with PBS and fixed with paraformaldehyde. The level of bound MBP-ECP was assessed by ELISA. The amount of MBP-ECP bound to cells without peptide treatment was set to 100%. The data shown are the means of triplicate experiments. B, Beas-2B cells were incubated with biotinylated peptides for 1 h at 4 °C. The amount of peptides bound to cells was assessed as for Fig. 3.

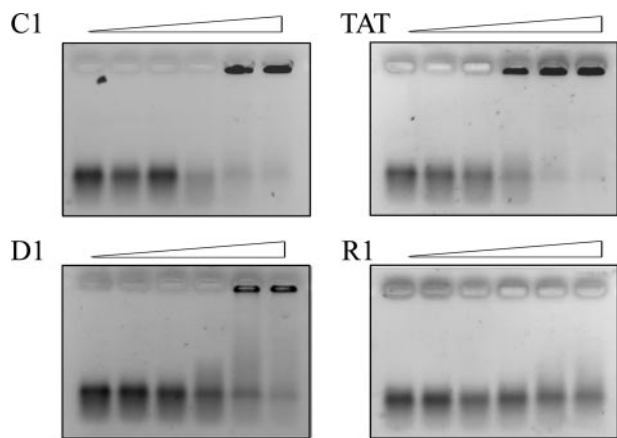


FIGURE 7. FACE analysis of synthetic peptides. AMAC-labeled LMWH were incubated without or with increasing concentrations of C1, HIV-TAT, D1 and R1 peptides at room temperature for 15 min. Samples were loaded and separated by agarose gel electrophoresis as described for Fig. 2.

FACE analysis. As expected, a decreased amount of free AMAC-LMWH signal was observed with increasing concentration of C1 peptide or TAT peptide (Fig. 7). In addition, D1 bound heparin as tightly as C1. Interestingly, although the peptide segment in the corresponding location of human RNase 1

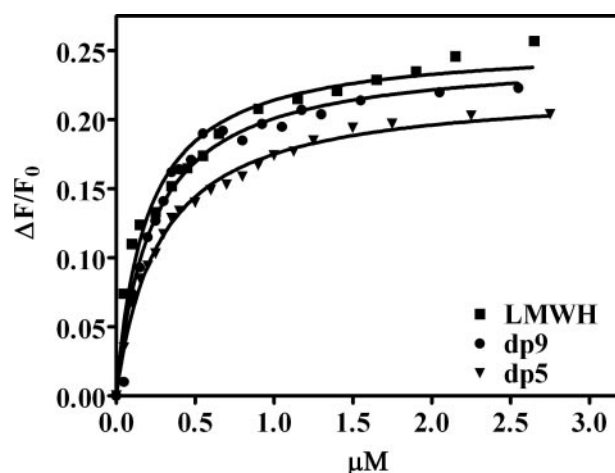


FIGURE 8. Titration profiles of synthetic peptides by synthetic heparin oligosaccharides. Titrations were carried out on 1 µM peptides in the presence of heparin oligosaccharides. The K_d of each peptide-oligosaccharide interaction was determined by measuring the fluorescence change of intrinsic tryptophan at 340 nm. Data were fitted to a single binding site curve using nonlinear least-squares analysis.

TABLE 2

Determination of dissociation constants (K_d) for C1 peptide with heparin oligosaccharides

Oligosaccharide	K_d
	nm
LMWH	192.0
dp9	229.1
dp5	274.8

also contains several positively charged residues, it did not bind heparin. Taken together, these results indicate that the RWRCK motif within loop L3 of ECP serves as a specific heparin binding site.

Characterization of Association between C1 and Heparin Oligosaccharides—The definitive heparin-binding activity of the C1 peptide was determined by ITFE, which clearly demonstrated that C1 has high affinity for heparin (Fig. 8). The K_d values obtained for LMWH, dp9, and dp5 binding to the C1 peptide were 192.0, 229.1, and 274.8 nM, respectively (Table 2), indicating that C1 bound tighter to longer heparin oligosaccharides.

Growth Inhibitory Effect of ECP—The inhibition of lymphocyte and mammalian cell growth by ECP has been reported (9, 30–32). As a physiological test, the cytotoxicity of ECP mt1 was monitored by MTT assay. As compared with wild-type ECP, ECP mt1 (R34A/W35A/R36A/K38A) exhibited a 2- to 3-fold increased IC_{50} value toward the Beas-2B cell line (Fig. 9 and Table 3). Thus, ECP mt1 without the key heparin binding sequence motif appears to be less cytotoxic than wild-type ECP, presumably due to weaker interaction with the cell surface, which in turn leads to decreased endocytosis.

Immunohistochemical Localization of ECP—To better understand the possible cellular targets of ECP, recombinant mature ECP was injected into the rat circulation through the tail vein. Immunohistochemical localization showed strong internalization of ECP in the tracheo- and bronchoepithelial cells 1 h post-injection (Fig. 10, A and B). ECP internalization was reduced when co-injection with heparin was carried out (Fig. 10C). For the neg-

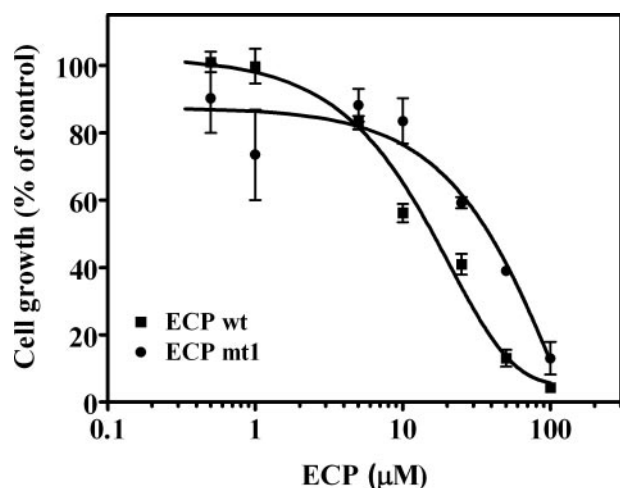


FIGURE 9. **Cytotoxicity of wild-type and mutant ECP.** Beas-2B cells were incubated with increasing concentrations of wild-type and mt1 ECP at 37 °C for 48 h, followed by the MTT assay as described under "Experimental Procedures." The error bars show standard deviation among triplicate experiments.

TABLE 3

IC₅₀ values for cytotoxic ECP

Protein	IC ₅₀
	μM
ECP wt ^a	16.05
ECP mt1	38.52

^a Data taken from Ref. 9.

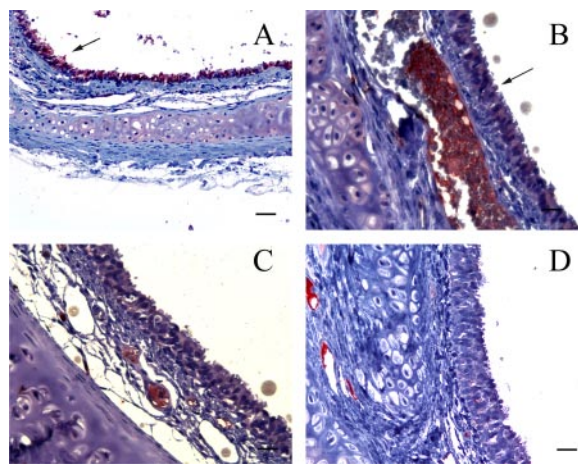


FIGURE 10. **Immunohistochemical localization of ECP using the Super Sensitive Non-Biotin HRP detection system.** Representative immunohistochemical staining patterns. ECP (red color) was detected in tracheo-epithelial cells (arrow) and cartilage cells 1 h post-iv injection (A and B). Decreased ECP signal was observed in tracheo-epithelial cells when ECP was co-injected with heparin (C). A control section of lung tissue subjected to MBP injection was used as a negative control (D). Magnification: A, 200×; B–D, 400×. Scale bars: 20 μm.

active control, no MBP signal was detected in tracheo- and bronchoepithelial cells (Fig. 10D), despite the MBP signal that could be detected in circulating blood (Fig. 10D).

DISCUSSION

Here we provide the first direct evidence that a linear heparin binding site within ECP contributes to its cellular binding and cytotoxicity. The peptide derived from this domain exhibits a substantial binding affinity to heparin.

The specific interaction with heparin mediated by the loop L3 region of ECP was analyzed by site-directed mutagenesis and synthetic peptides to determine the dissociation constant. We have shown that heparin dp5 binds to ECP with a K_d of 139.6 nM. Increased ECP binding affinity with increasing heparin oligosaccharide chain length was observed, indicating that longer heparin chains with an additional structural motif might strengthen polysaccharide-protein interactions. The mutagenesis study revealed that ECP mt1 (R34A/W35A/R36A/K38A), lacking the crucial heparin binding sequence motif, had reduced heparin-binding activity and was thus less cytotoxic than wild-type ECP.

ITFE demonstrated that ECP mt1 had reduced heparin-binding activity. To further show direct heparin-binding activity of the loop L3 in ECP, the C1 and D1 peptides spanning residues 32–41 of ECP and EDN, respectively, were synthesized. Cell ELISA analysis revealed that C1 and D1 peptides interfered molecular interaction between ECP and cell surface HSPG. Studies of heparin binding by FACE showed that ECP C1 and EDN D1 bound as tightly to heparin as HIV-TAT peptide. On the other hand, the R1 peptide derived from RNase1, with positive charge density similar to C1, showed much lower affinity toward heparin, indicating that specific heparin-binding activity of C1 resulted not only from the positive charges but also from the amino acid sequence. Furthermore, in ITFE binding assays, C1 had high binding capacity for heparin, indicating that the heparin binding site in ECP is indeed located within the loop L3 region connecting helix $\alpha 2$ and strand $\beta 1$.

It has been shown that *N*-bromosuccinimide-modified ECP elutes from heparin chromatography at a slightly lower concentration of NaCl than unmodified ECP, indicating the contribution of tryptophan residues (Trp-10 and Trp-35) to heparin binding (32). The cellular binding activity of ECP W10A was only slightly decreased (<10%) as compared with that of wild-type ECP, whereas ECP W35A exhibited 50% lower binding, strongly indicating the involvement of Trp-35 in heparin binding.³ In this study, Trp-35 together with the positively charged residues in loop L3 of ECP are likely to constitute a novel heparin-binding motif and, thus, are responsible for the heparin-binding activity.

The heparin-binding activity of ECP mt1 was reduced 5-fold as compared with that of wild-type ECP, suggesting that residues Arg-34, Trp-35, Arg-36, and Lys-38 contribute to the overall free energy of the heparin-ECP interaction. However, this mutant still retained some heparin-binding ability. Because there are 19 arginine residues on the surface of ECP, some of which may form an extended binding surface together with the loop L3, detailed analysis of the contribution of each positively charged residue remains to be determined. Interestingly, the putative binding site identified in this study shares common features with that of EDN, indicating that the loop L3 of eosinophil RNases may contribute to their shared functions. These results suggest that the heparin binding and uptake mechanism of EDN are likely to be similar to those of ECP.

³ T.-c. Fan, S.-l. Fang, C.-s. Hwang, C.-y. Hsu, X.-a. Lu, S.-c. Hung, S.-C. Lin, and M. D.-T. Chang, unpublished data.

The accumulation of a high concentration of ECP on the cell surface through interaction with HSPGs likely results in membrane perturbation and subsequent cytotoxicity. Immunohistochemical staining showed that ECP had high affinity for tracheo- and bronchoepithelial cells. Although HSPGs are ubiquitously expressed on all kinds of cell surfaces and in the extracellular matrix, the HS disaccharide composition seems to be tissue-specific (33, 34). The HS disaccharide composition and the overall degree of sulfation of murine tissues have been analyzed. The HS fingerprint has shown that lung tissue contains relatively high overall 2-O-sulfation due to the presence of 2-O-sulfated glucuronic acid-containing disaccharides (34). Moreover, HS is presented in block structures, consisting of highly sulfated and non-sulfated domains. It is possible that HSs of tracheo- and bronchoepithelial cells have unique characteristics that serve as the principle receptor or co-receptor for ECP. The most prominent example is the requirement of contiguous stretches of the disaccharide 2-O-sulfated iduronic acid N-sulfated glucosamine for efficient interaction with fibroblast growth factor-2, and sulfation at the 6-O position of heparin may be required for cell signaling (15, 26).

In summary, we have determined that pentasaccharides are the minimal sugar motif that can bind to ECP. The specific heparin binding site located within the loop L3 of ECP is critical for its physiological function. Our results may assist in determining the physiological role of ECP *in vivo* and further rational design of heparin mimetics to interfere with the cellular interactions of cytotoxic ECP.

REFERENCES

- Boix, E., Nikolovski, Z., Moiseyev, G. P., Rosenberg, H. F., Cuchillo, C. M., and Nogues, M. V. (1999) *J. Biol. Chem.* **274**, 15605–15614
- Molina, H. A., Kierszenbaum, F., Hamann, K. J., and Gleich, G. J. (1988) *Am. J. Trop. Med. Hyg.* **38**, 327–334
- Lehrer, R., Szklarek, D., Barton, A., Ganz, T., Hamann, K., and Gleich, G. (1989) *J. Immunol.* **142**, 4428–4434
- Domachowske, J. B., Dyer, K. D., Adams, A. G., Leto, T. L., and Rosenberg, H. F. (1998) *Nucleic Acids Res.* **26**, 3358–3363
- Gleich, G. J. (2000) *J. Allergy Clin. Immunol.* **105**, 651–663
- Young, J., Peterson, C., Venge, P., and Cohn, Z. (1986) *Nature* **321**, 613–616
- Carreras, E., Boix, E., Navarro, S., Rosenberg, H. F., Cuchillo, C. M., and Nogues, M. V. (2005) *Mol. Cell. Biochem.* **272**, 1–7
- Carreras, E., Boix, E., Rosenberg, H. F., Cuchillo, C. M., and Nogues, M. V. (2003) *Biochemistry* **42**, 6636–6644
- Fan, T. C., Chang, H. T., Chen, I. W., Wang, H. Y., and Chang, M. D. (2007) *Traffic* **8**, 1778–1795
- Capila, I., and Linhardt, R. J. (2002) *Angew. Chem. Int. Ed. Engl.* **41**, 391–412
- Tumova, S., Woods, A., and Couchman, J. R. (2000) *Int. J. Biochem. Cell Biol.* **32**, 269–288
- Mallorqui-Fernandez, G., Pous, J., Peracaula, R., Aymami, J., Maeda, T., Tada, H., Yamada, H., Seno, M., Llorens, R. d., Gomis-Ruth, F. X., and Coll, M. (2000) *J. Mol. Biol.* **300**, 1297–1307
- Hileman, R. E., Fromm, J. R., Weiler, J. M., and Linhardt, R. J. (1998) *BioEssays* **20**, 156–167
- Cardin, A. D., and Weintraub, H. J. (1989) *Arteriosclerosis* **9**, 21–32
- Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) *J. Biol. Chem.* **268**, 23906–23914
- Lee, C. J., Lu, X. A., Kulkarni, S. S., Wen, Y. S., and Hung, S. C. (2004) *J. Am. Chem. Soc.* **126**, 476–477
- Calabro, A., Benavides, M., Tammi, M., Hascall, V. C., and Midura, R. J. (2000) *Glycobiology* **10**, 273–281
- Holmes, O., Pillozzi, S., Deakin, J. A., Carafoli, F., Kemp, L., Butler, P. J., Lyon, M., and Gherardi, E. (2007) *J. Mol. Biol.* **367**, 395–408
- Venge, P., and Bystrom, J. (1998) *Int. J. Biochem. Cell Biol.* **30**, 433–437
- Liang, C. T., Chueh, L. L., Pang, V. F., Zhuo, Y. X., Liang, S. C., Yu, C. K., Chiang, H., Lee, C. C., and Liu, C. H. (2007) *J. Comp. Pathol.* **136**, 57–64
- Gleich, G. J., Loegering, D. A., Bell, M. P., Checkel, J. L., Ackerman, S. J., and McKean, D. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3146–3150
- Joyce, J. A., Freeman, C., Meyer-Morse, N., Parish, C. R., and Hanahan, D. (2005) *Oncogene* **24**, 4037–4051
- Parish, C. R., Freeman, C., Brown, K. J., Francis, D. J., and Cowden, W. B. (1999) *Cancer Res.* **59**, 3433–3441
- Yu, G., Gunay, N. S., Linhardt, R. J., Toida, T., Fareed, J., Hoppensteadt, D. A., Shadid, H., Ferro, V., Li, C., Fewings, K., Palermo, M. C., and Podger, D. (2002) *Eur. J. Med. Chem.* **37**, 783–791
- Ferro, V., Li, C., Fewings, K., Palermo, M. C., Linhardt, R. J., and Toida, T. (2002) *Carbohydr. Res.* **337**, 139–146
- Raman, R., Sasisekharan, V., and Sasisekharan, R. (2005) *Chem. Biol.* **12**, 267–277
- Lau, E. K., Paavola, C. D., Johnson, Z., Gaudry, J. P., Geretti, E., Borlat, F., Kungl, A. J., Proudfoot, A. E., and Handel, T. M. (2004) *J. Biol. Chem.* **279**, 22294–22305
- Vives, E., Brodin, P., and Lebleu, B. (1997) *J. Biol. Chem.* **272**, 16010–16017
- Ziegler, A., and Seelig, J. (2004) *Biophys. J.* **86**, 254–263
- Peterson, C. G., Skoog, V., and Venge, P. (1986) *Immunobiology* **171**, 1–13
- Kimata, H., Yoshida, A., Ishioka, C., Jiang, Y., and Mikawa, H. (1992) *Cell. Immunol.* **141**, 422–432
- Maeda, T., Kitazoe, M., Tada, H., Llorens, R. d., Salomon, D. S., Ueda, M., Yamada, H., and Seno, M. (2002) *Eur. J. Biochem.* **269**, 307–316
- Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem.* **271**, 17804–17810
- Ledin, J., Staatz, W., Li, J. P., Gotte, M., Selleck, S., Kjellen, L., and Spillmann, D. (2004) *J. Biol. Chem.* **279**, 42732–42741