Role of the Proposed Serpin-Enzyme Complex Receptor Recognition Site in Binding and Internalization of Thrombin-Heparin Cofactor II Complexes by Hepatocytes

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Several serpin-enzyme complexes bind to a receptor on hepatocytes that mediates their endocytosis and lysosomal degradation. Joslin et al. (Joslin, G., Fallon, R. J., Bullock, J., Adams, S. P., and Perlmutter, D. H. (1991) J. Biol. Chem. 266, 11282-11288) proposed that a sequence near the C-terminal end of the serpin (e.g. FVFLM in α1-antitrypsin) binds to the serpin-enzyme complex receptor (SEC receptor). In experiments with synthetic peptides, they found that substitution of alanine at the fourth or fifth position in this sequence reduced the affinity of peptide binding to Hep G2 cells. To test this hypothesis, we constructed five recombinant HCII variants, F456A, L457A, F458A, L459A, and I460A. To test the hypothesis that the corresponding sequence in heparin cofactor II (HCII), FFLLI (residues 456-460), mediates binding and uptake of the thrombin-HCII complex by Hep G2 cells, we constructed five recombinant HCII variants, F456A, L457A, F458A, L459A, and I460A. At 4°C, the 125I-thrombin-HCII (native) complex bound reversibly to 0.6-2.6 × 10^6 sites per Hep G2 cell with a K_d of 19-32 nM. Binding was inhibited by excess unlabeled thrombin-HCII (native), thrombin-antithrombin, or elastase-α1-antitrypsin, but not by free HCII or thrombin, which is consistent with the reported specificity of the SEC receptor. However, complexes of thrombin with each of the HCII variants inhibited binding as effectively as the complex with native HCII. Competitive binding experiments with various concentrations of unlabeled thrombin-HCII (native) or thrombin-HCII (1460A) indicated that these complexes bind to Hep G2 cells with equal affinity. At 37°C, complexes of 125I-thrombin with each of the five HCII variants were internalized and degraded at the same rate as the complex with native HCII. Our data suggest that the pentapeptide FFLLI in HCII is not involved in binding, internalization, and degradation of thrombin-HCII complexes by Hep G2 cells.

The serpins comprise a large family of homologous proteins that are widely distributed in nature (1, 2). Many serpins inhibit extracellular serine proteases that participate in hemostasis, fibrinolysis, or inflammation. Heparin cofactor II (HCII) is a serpin found in mammalian plasma that inhibits thrombin >1000 times more rapidly in the presence of heparin or dermatan sulfate than in the absence of a glycosaminoglycan (3). Like other serpins, HCII inhibit thrombin by forming a stable 1:1 complex with the protease. Thrombin-HCII and several other serpin-enzyme complexes bind to a receptor on the surface of hepatocytes (termed the "SEC receptor"), which mediates endocytosis and lysosomal degradation of the complexes (4, 5). Thus, the SEC receptor may be involved in clearance of serpin-enzyme complexes from the circulation.

The serpin-enzyme complexes elastase-α1-antitrypsin, cathespin G-α1-antichymotrypsin, thrombin-antithrombin, and thrombin-HCII compete with one another for binding to the SEC receptor on human hepatoma (Hep G2) cells (6). The uncomplexed serpins and proteases, however, do not bind to the receptor. Joslin et al. (7) proposed that a pentapeptide sequence near the C-terminal end of the serpin (i.e. FVFLM) in α1-antitrypsin, FLMII in α1-antichymotrypsin, FLVFI in antithrombin, and FFLLI in HCII) becomes accessible during complex formation and mediates binding of the complex to the SEC receptor. In support of this hypothesis, they showed that binding of 125I-trypsin-α1-antitrypsin to Hep G2 cells was competitively inhibited by the synthetic peptide FVFLM. Additional experiments suggested that interaction of peptides with the SEC receptor was sequence-specific. In particular, FVFLA did not inhibit 125I-trypsin-α1-antitrypsin binding, and FVFAM was a relatively weak inhibitor of binding in comparison with FVFLM (7). Accordingly, the presence of alanine at the corresponding positions in a serpin would be predicted to disrupt binding of the serpin-enzyme complex to the SEC receptor.

To test the hypothesis that FFLLI (positions 456-460) in HCII mediates binding to the SEC receptor, we constructed five recombinant HCII (rHCII) variants, each containing an alanine substitution at one of the positions in the pentapeptide sequence. In contrast to predictions made from the synthetic peptide experiments of Joslin et al. (7), we found that none of the mutations in the proposed SEC receptor recognition site of HCII diminished the binding, internalization, and degradation of thrombin-HCII complexes by Hep G2 cells.

EXPERIMENTAL PROCEDURES

Materials—HCII and antithrombin were purified from human plasma as described previously (8). Human α1-antitrypsin and human leukocyte elastase were obtained from Alexis Biochemicals (San Diego, CA) and human α-thrombin from HaematoLogic Technologies (Essex Junction, VT). Thrombin was inactivated with o-phenylalanilinyl-o-prolyl-o-arginine methyl chloromethyl ketone (PPACK, Calbiochem) as described by Kettner and Shaw (9). Porcine intestinal heparin and porcine skin dermatan sulfate were purchased from Sigma; the dermatan sulfate combinator HCII; SEC receptor, serpin-enzyme complex receptor; PPACK, o-phenylalanilinyl-o-prolyl-o-arginine methyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis.
was treated with nitrous acid to degrade contaminating heparin or heparan sulfate (8). Other materials were obtained from the following sources: papain, phenylmethylsulfonyl fluoride, isopropyl β-D-thiogalactopyranoside, and deoxyribonuclease-I from Sigma; polyethylene glycol 8000 from Union Carbide (Danbury, CT); Chromazym TH (tosyl-Gly-Pro-Arg-p-nitroanilide) from Boehringer Mannheim; heparin-Sepharose CL-4B and S-200 from Pharmacia Biotech Inc.; restriction enzymes from New England Biolabs (Beverly, MA); and medium for high density bacterial culture from BTO 101 (Vista, CA).

Determination of Protein Concentration—HCII and antithrombin concentrations were determined from the absorbance at 280 nm, using extinction coefficients of 1.17 mg cm⁻² and 3.23 mg cm⁻², respectively. Concentrations of other proteins and serpin-enzyme complexes were determined using the protein assay kit from Bio-Rad standardized with bovine serum albumin.

SDS-PAGE and Autoradiography—Protein samples were analyzed by SDS-PAGE on 7.5% or 10% gels under reducing conditions according to the method of Laemmli (12). Autoradiography was performed as described previously (8).

Expression and Purification of HCII—The full-length HCII cDNA (13) was inserted between the NcoI and BamHI sites of the pET-3d expression vector (Novagen, Madison, WI). Oligonucleotide-directed mutagenesis was performed with the Transformer Site-directed Mutagenesis Kit (Clontech, Palo Alto, CA) using oligonucleotides synthesized in the Nucleic Acid Chemistry Laboratory at Washington University. The mutations and ligation sites were verified by dideoxynucleotide sequencing (14).

Plasmid vectors were electroporated into E. coli BL21 (DE3)pLYS5 cells for expression. The cells were grown to an optical density (600 nm) of 5–6 in a BioFlo III high-density fermentor (New Brunswick Scientific, Edison, NJ) at 37 °C and then induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside for expression. The cells were grown to an optical density (600 nm) of 1.17 mg cm⁻² and 3.23 mg cm⁻², respectively. Concentrations of other proteins and serpin-enzyme complexes were determined using the protein assay kit from Bio-Rad standardized with bovine serum albumin.

Binding of 125I-Thrombin-HCII to Hep G2 Cells—Before we could investigate whether mutations in HCII affect the interaction of thrombin-HCII complexes with the SEC receptor, it was necessary to characterize the binding of 125I-thrombin-HCII complexes to Hep G2 cells in greater detail (17). To this end, we prepared radiolabeled thrombin-HCII complexes by incubating 125I-thrombin with purified plasma HCII in the absence of a glycosaminoglycan. The complexes were separated from free 125I-thrombin and HCII by heparin-Sepharose chromatography (see “Experimental Procedures”) and analyzed by SDS-PAGE and autoradiography. Fig. 1 (panel A) indicates that almost all of the radioactivity was present in an SDS-stable complex of the appropriate molecular weight (~96,000) (10).

Binding studies were performed at 4 °C to minimize internalization and degradation of the ligand. The time course of binding of 10 nM 125I-thrombin-HCII to Hep G2 cells is shown in Fig. 1 (panel B). Maximal specific binding was attained after a 2-h incubation and represented ~1.5% of the total radioactivity added to the cells (closed circles). Analysis of the medium and the washed cell monolayer by SDS-PAGE and autoradiography indicated that the 125I-thrombin-HCII complex remained intact and that the bound radioactivity did not represent a labeled contaminant (Fig. 1, panel C). To determine the reversibility of binding after 2 h at 4 °C, the cells were washed and then incubated with a 50-fold molar excess of unlabeled thrombin-HCII. During a subsequent 2-h incubation, the bound radioactivity progressively decreased to the level of nonspecific binding (Fig. 1, panel B, closed triangles).

Specific binding became saturated over the concentration range of 5–80 nM 125I-thrombin-HCII, whereas nonspecific binding increased linearly (Fig. 2, panel A). Scatchard analysis

RESULTS

Equilibrium Binding of 125I-Thrombin-HCII Complexes to Hep G2 Cells—As we could investigate whether mutations in HCII affect the interaction of thrombin-HCII complexes with the SEC receptor, it was necessary to characterize the binding of 125I-thrombin-HCII complexes to Hep G2 cells in greater detail (17). To this end, we prepared radiolabeled thrombin-HCII complexes by incubating 125I-thrombin with purified plasma HCII in the absence of a glycosaminoglycan. The complexes were separated from free 125I-thrombin and HCII by heparin-Sepharose chromatography (see “Experimental Procedures”) and analyzed by SDS-PAGE and autoradiography. Fig. 1 (panel A) indicates that almost all of the radioactivity was present in an SDS-stable complex of the appropriate molecular weight (~96,000) (10).

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Specific binding became saturated over the concentration range of 5–80 nM 125I-thrombin-HCII, whereas nonspecific binding increased linearly (Fig. 2, panel A). Scatchard analysis
suggested a single class of binding sites (≈0.6 × 10^6 sites per Hep G2 cell) with a dissociation constant ($K_d$) of 19 nM (Fig. 2, panel B). In an experiment with a different batch of Hep G2 cells, −2.6 × 10^6 molecules of complex bound per cell with a $K_d$ of 32 nM (data not shown).

Further Characterization of the Binding Site for 125I-Thrombin-HCII on Hep G2 Cells—To confirm that we were detecting binding of 125I-thrombin-HCII to the SEC receptor of Hep G2 cells, as operationally defined in previous studies, we determined binding in the presence of a 40-fold molar excess of various competitors. SDS-PAGE of the proteins used as competitors in this experiment is shown in Fig. 3 (panel A). No inhibition of binding was observed in the presence of HCII, antithrombin, α1-antitrypsin, or thrombin inactivated with PPACK (Fig. 3, panel B). By contrast, purified thrombin-HCII, thrombin-antithrombin, and elastase-α1-antitrypsin complexes inhibited binding of the 125I-thrombin-HCII complex by 60–70%. These results are consistent with previous reports that the SEC receptor does not recognize the free serpin or its target protease and that several serpin–enzyme complexes compete with one another for binding to Hep G2 cells (4, 6). Preparations of HCII, antithrombin, and α1-antitrypsin inactivated by cleavage of the reactive site loop also inhibited binding of 125I-thrombin-HCII to a modest degree (see “Discussion”).

Expression of Mutant rHCII—To investigate the proposed SEC receptor recognition site, we constructed the HCII variants shown in Fig. 4 (panel A). Each form of rHCII was expressed in E. coli, purified, and analyzed by SDS-PAGE. As shown in Fig. 4 (panel B), the mutant proteins were identical in size to native rHCII and had an apparent $M_r$ of −58,000. The molecular weight of rHCII is less than that of human plasma HCII because of the absence of asparagine-linked glycosylation in E. coli.

We determined second-order rate constants for inhibition of thrombin to assess the functional integrity of the mutant proteins. As indicated in Table I, the rate constants for inhibition of thrombin by each of the variants in the presence of heparin or dermatan sulfate were essentially identical to that of native HCII (−1.5 × 10^9 M⁻¹min⁻¹) and were approximately 5000 times higher than the rate constants determined in the absence of a glycosaminoglycan (−3.0 × 10^6 M⁻¹min⁻¹). Furthermore, the affinities of the native and mutant rHCII for heparin were indistinguishable as determined by chromatography on heparin-Sepharose (data not shown).

Inhibition of 125I-Thrombin-HCII Binding to Hep G2 Cells by Thrombin-rHCII Complexes—Competitive inhibition studies were performed to determine whether complexes of thrombin with the rHCII variants bind to the SEC receptor. Complexes prepared by incubating the rHCII variants in Fig. 4 with thrombin were isolated by heparin-Sepharose chromatography. The final preparations were homogeneous by SDS-PAGE (data not shown). In control experiments, binding of 125I-thrombin-HCII to Hep G2 cells was not inhibited by a 40-fold molar excess of unlabeled native rHCII or by any of the rHCII variants (Fig. 5). By contrast, the thrombin-rHCII (native) complex inhibited binding −70%, and binding was inhibited to an equal extent by complexes of thrombin with each of the five rHCII variants. The result obtained with thrombin-rHCII(I460A) was particularly unexpected, because substitution of alanine at the corresponding position of a synthetic pentapeptide almost completely abrogated its binding to the SEC receptor (7). To test for
A subtle difference in the $K_d$ values for binding of thrombin-rHCII(I460A) and thrombin-rHCII(native) to Hep G2 cells, we performed competitive binding assays with lower concentrations of both unlabeled ligands. The results (Fig. 6) indicate that thrombin-rHCII(I460A) and thrombin-rHCII(native) have similar affinities for the SEC receptor.

Internalization and Degradation of $^{125}$I-Thrombin-rHCII Complexes—We prepared complexes of $^{125}$I-thrombin with native or mutant rHCII as described above, incubated the complexes for 2–6 h with Hep G2 cells at 37 °C, and measured the accumulation of trichloroacetic acid-soluble degradation products in the culture medium. Fig. 7 (panel A, closed circles) shows the time-dependent uptake and degradation of $^{125}$I-thrombin-rHCII(native) complexes. Uptake and degradation were not inhibited by excess unlabeled PPACK-thrombin (open circles) or by HCII (closed squares) but were partially inhibited by thrombin-HCII complexes (open squares), which is consistent with involvement of the SEC receptor (5). Uptake and degradation were inhibited ~75% by 200 μM chloroquine (closed triangles), which suggests that degradation occurs via the lysosomal pathway. Fig. 7 (panel B) shows a separate experiment in which complexes prepared with native or mutant rHCII were incubated for 6 h at 37 °C with Hep G2 cells in the absence or presence of chloroquine. Uptake and lysosomal degradation of complexes containing each of the mutant rHCII$s$ occurred at the same rate as that of the $^{125}$I-thrombin-rHCII(native) complex.

**DISCUSSION**

The observation that trypsin-α1-antitrypsin complexes are eliminated from mouse plasma in vivo ~5–10 times more rapidly than free α1-antitrypsin first suggested the existence of a specific clearance mechanism for serpin-enzyme complexes (23). Subsequent studies demonstrated uptake of radiolabeled serpin-enzyme complexes by hepatocytes (24, 25). Cross-competition experiments in vivo suggested that complexes containing α1-antitrypsin, α1-antichymotrypsin, antithrombin, or heparin cofactor II are cleared by the same hepatic receptor (26, 27), whereas α2-antiplasmin-protease complexes are eliminated by a different mechanism (28). Rapid clearance is independent of the protease used to form the complex, which implies that the receptor recognizes a change in the structure of the serpin that follows complex formation. Receptors with similar ligand specificity have also been identified on neutrophils (29, 30) and monocytes. The elastase-α1-antitrypsin complex, but not free α1-antitrypsin, is chemotactic for neutrophils (29, 30) and stimulates biosynthesis of α1-antitrypsin in monocytes and macrophages (31, 32).

Banda et al. (33) reported that the C-terminal 4.2-kDa peptide derived from cleavage of α1-antitrypsin at the reactive site stimulates neutrophil chemotaxis. Similarly, Perlmutter and co-workers (4, 7) showed that biosynthesis of α1-antitrypsin in Hep G2 cells and monocytes is stimulated by synthetic peptides based on the C-terminal sequence of α1-antitrypsin. One of
proteases. Subsequently, these investigators showed that the 125I-glycine-1-antitrypsin-elastase complex binds to Hep G2 cells with a Kd of 30–40 nM and then undergoes internalization and lysosomal degradation (5). Since binding and internalization were competitively inhibited by unlabeled peptide 105Y, elastase-α1-antitrypsin, or trypsin-α1-antitrypsin, they suggested that a single receptor on Hep G2 cells, termed the SEC receptor, is involved both in receptor-mediated endocytosis and in triggering a signal transduction pathway that leads to increased α1-antitrypsin gene expression.

In a subsequent report, Joslin et al. (7) proposed that the pentapeptide FVFLM (residues 370–374) of α1-antitrypsin and the corresponding sequences in other serpins (FLMI in α1-antichymotrypsin, FLVFI in antithrombin, and FFLFI in HCII) mediate binding of the serpin-enzyme complexes to the SEC receptor. They demonstrated that binding of 125I-105Y or 125I-trypsin-α1-antitrypsin to Hep G2 cells was inhibited by excess unlabeled FVFLM. Additional experiments indicated that the pentapeptide FVYLI (contained in peptide 105Y), but not the truncated peptides FVYL or VYLI, could function as a competitive inhibitor of binding. Various amino acid substitutions in the synthetic peptide FVFLM suggested that binding to the SEC receptor is somewhat sequence-specific. Peptides in which any one of the first three amino acids was replaced with alanine (i.e., AVFML, FAFLM, and FVALM) inhibited binding of 125I-trypsin-α1-antitrypsin to Hep G2 cells. By contrast, FVFAL was almost completely inactive in the competitive binding assay, and FVFAM had only moderate activity in comparison with that of the native peptide FVFLM (7).

We designed the current study as a critical test of the hypothesis that the pentapeptide FVFLM (residues 370–374) of α1-antitrypsin binds to the SEC receptor. To achieve this, we tested the ability of other pentapeptides derived from α1-antitrypsin and other serpins to compete with 125I-FVFLM for binding to Hep G2 cells.

Table 1: Second-order rate constants for inhibition of thrombin by rHCII variants

| rHCII | No GAG | + Heparin (5 μg/ml) | + Dermatan sulfate (20 μg/ml) |
|-------|--------|---------------------|-----------------------------|
| Native| 3.2 × 10^4 | 1.5 × 10^6 | 1.6 × 10^6 |
| F456A | 1.0 × 10^4 | 1.0 × 10^6 | 1.1 × 10^6 |
| L457A | 2.9 × 10^4 | 1.1 × 10^6 | 1.8 × 10^6 |
| F459A | 2.9 × 10^4 | 1.4 × 10^6 | 1.3 × 10^6 |
| L459A | 1.7 × 10^4 | 1.7 × 10^6 | 1.7 × 10^6 |
| I460A | 1.9 × 10^4 | 1.8 × 10^6 | 1.8 × 10^6 |

* rHCII, recombinant HCII; GAG, glycosaminoglycan.

*Tyrosine was substituted for a phenylalanine in the α1-antitrypsin sequence to allow radioiodination of the peptide, and the C-terminal methionine was replaced by isoleucine to facilitate chemical synthesis.

**FIG. 5. Inhibition of 125I-thrombin-HCII binding to Hep G2 cells by mutant rHCII-thrombin complexes.** Hep G2 cells were incubated for 2 h at 4°C with 10 nM 125I-thrombin-HCII in the presence of 400 nM unlabeled competitor, and the bound radioactivity was determined as described under "Experimental Procedures." The results are expressed as a percentage of the binding observed in the absence of a competitor and are plotted as the mean ± S.D. of triplicate determinations.

**FIG. 6. Inhibition of 125I-thrombin-HCII binding by thrombin-rHCII(native) and thrombin-rHCII(I460A).** Incubations were performed as in Fig. 5, except that various concentrations of unlabeled competitor were used. Each point represents the mean of duplicate determinations. ○, thrombin-rHCII(native); ●, thrombin-rHCII(I460A).
pothesis that FLFLI (residues 456–460) mediates binding and endocytosis of the thrombin-HCII complex by Hep G2 cells. First, we showed that purified complexes prepared with $^{125}$I-thrombin and plasma HCII bind reversibly at 4°C to 0.6–2.6 $\times$ 10$^7$ sites per Hep G2 cell with a $K_d$ of 19–32 nM (Figs. 1 and 2), which agrees closely with the $K_d$ values reported for binding of peptide $^{125}$I-105Y or $^{125}$I-FLFLI to Hep G2 cells. However, excess unlabeled elastase-$\alpha$1-antitrypsin inhibited only ~60% of the binding of $^{125}$I-105Y to Hep G2 cells (5), which raises the possibility that the peptide binds to more than one component on the cell surface.

Next, we demonstrated that binding of $^{125}$I-thrombin-HCII is inhibited by excess unlabeled thrombin-HCII and other serpin-envelope complexes but not by free HCII or active site-blocked thrombin (PPACK-thrombin) (Fig. 3). These results are consistent with the ligand specificity of the SEC receptor as previously defined. Proteolytically inactivated forms of HCII, antithrombin, and $\alpha$1-antitrypsin inhibited binding of $^{125}$I-thrombin-HCII less well than equimolar concentrations of the corresponding serpin-envelope complexes (Fig. 3), in agreement with the observation that cleaved serpins are not cleared rapidly from the circulation (19). We also demonstrated that endocytosis and degradation of $^{125}$I-thrombin-HCII at 37°C is partially inhibited by excess unlabeled thrombin-HCII (Fig. 7, panel A) but not by free HCII or PPACK-thrombin, consistent with involvement of the SEC receptor in this process.

Finally, we constructed a series of alanine substitutions in the proposed SEC receptor recognition site of rHCII (Fig. 4) and showed that the mutant proteins are expressed as fully active inhibitors (Table I). As predicted from the results obtained with synthetic peptides (7), mutation of Phe$^{456}$, Leu$^{457}$, or Phe$^{458}$ does not affect binding or endocytosis of $^{125}$I-thrombin-HCII by Hep G2 cells. By contrast, the synthetic peptide experiments predicted that mutation of Ile$^{460}$ to alanine would greatly reduce the binding affinity of the thrombin-rHCII(1460A) complex and slow the rate of receptor-mediated endocytosis (7). The data in Figs. 5–7, however, indicate that this mutation has no effect on either process. Likewise, replacement of Leu$^{459}$ with alanine has no effect, although a pentapeptide with alanine in the corresponding position had a moderately decreased affinity for Hep G2 cells (7).

Our data do not support the hypothesis that the pentapeptide FLFLI in HCII (or, by implication, the corresponding sequences in other serpins) mediates binding, internalization, and degradation of the serpin-envelope complex by Hep G2 cells. Schulze et al. (34) also have questioned involvement of the pentapeptide in these processes, citing crystallographic evidence that this sequence is buried in the hydrophobic core of the serpin. Based on the assumption that peptide 105Y mimics binding of serpin-envelope complexes to Hep G2 cells, Perlmutter (35) has proposed that the ligand-binding subunit of the SEC receptor is an 80-kDa polypeptide identified by photoaffinity cross-linking of radioiodinated 105Y to Hep G2 cell membranes. However, Kounnas et al. (36) recently demonstrated that the low density lipoprotein receptor-related protein, the ligand-binding subunit of which is 515 kDa in size, is responsible for endocytosis and degradation of thrombin-HCII, thrombin-antithrombin, and trypsin-$\alpha$1-antitrypsin complexes by Hep G2 cells and for the in vivo clearance of $^{125}$I-thrombin-antithrombin complexes in rats. Whether the low density lipoprotein receptor-related protein or the 80-kDa polypeptide participates in the signaling events attributed to the SEC receptor remains to be determined.

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