

# A Unique Loop Extension in the Serine Protease Domain of Haptoglobin Is Essential for CD163 Recognition of the Haptoglobin-Hemoglobin Complex\*

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Haptoglobin and haptoglobin-related protein are homologous hemoglobin-binding proteins consisting of a complement control repeat ( $\alpha$ -chain) and a serine protease domain ( $\beta$ -chain). Haptoglobin-hemoglobin complex formation promotes high affinity binding of hemoglobin to the macrophage scavenger receptor CD163 leading to endocytosis and degradation of the haptoglobin-hemoglobin complex. In contrast, complex formation between haptoglobin-related protein and hemoglobin does not promote high affinity interaction with CD163. To define structural components of haptoglobin important for CD163 recognition, we exploited this functional difference to design and analyze recombinant haptoglobin/haptoglobin-related protein chimeras complexed to hemoglobin. These data revealed that only the  $\beta$ -chain of haptoglobin is involved in receptor recognition. Substitution of 4 closely spaced amino acid residues of the haptoglobin  $\beta$ -chain (valine 259, glutamate 261, lysine 262, and threonine 264) abrogated the high affinity receptor binding. The 4 residues are encompassed by a part of the primary structure not present in other serine protease domain proteins. Structural modeling based on the well characterized serine protease domain fold suggests that this sequence represents a loop extension unique for haptoglobin and haptoglobin-related protein. A synthetic peptide representing the haptoglobin loop sequence exhibited a pronounced inhibitory effect on receptor binding of haptoglobin-hemoglobin.

Haptoglobin (Hp)<sup>2</sup> is an acute phase protein that circulates in high amounts (0.45–3 mg/ml) in plasma. The primary function of Hp is to capture hemoglobin (Hb) leaking into plasma from ruptured erythrocytes and precursors during intravascular hemolysis. The resulting high affinity complex between Hp and Hb is cleared from the circulation by receptor-mediated endocytosis via the acute phase-regulated monocyte/macrophage-specific protein CD163 (1), which binds Hp-Hb by its scavenger receptor cysteine-rich domain region (2). This Hb clearance mechanism protects against inexpedient toxic effects caused by the oxidative iron-containing heme prosthetic group present in Hb (3).

Hp is synthesized as a single polypeptide precursor (proHp) that is proteolytically processed to form separate  $\alpha$ - and  $\beta$ -chains representing a complement control repeat and a serine protease domain, respectively. The  $\alpha$ - and  $\beta$ -chains are linked by a single disulfide bond. The Hp protein of most mammals is composed of two ( $\alpha\beta$ )-monomers linked together via a disulfide bond between the two  $\alpha$ -chains generating an ( $\alpha\beta$ )<sub>2</sub> structure (termed Hp1-1 in humans). However, three Hp phenotypes exist in humans due to the presence of two Hp gene alleles, designated Hp1 and Hp2. The Hp2 allele, which arose by an intragenic duplication of the Hp1 allele, encodes a slightly larger  $\alpha$ -chain but is otherwise identical to the Hp1 allele. Because the cysteine residue connecting the  $\alpha$ -chains is duplicated in the encoded Hp2 protein, the Hp2-1 and Hp2-2 phenotypes display a spectrum of various Hp ( $\alpha\beta$ )-multimers (4, 5).

Due to an Hp gene triplication in the late evolution, primates also express the haptoglobin-related protein (Hpr), which shares 91% sequence identity with the Hp1 protein (6, 7). Hpr circulates in plasma as a component of two complexes, a subclass of high density lipoprotein particles (~500 kDa) known as trypanosome lytic factor 1 and a high molecular mass ( $\geq 1000$  kDa), lipid-poor protein complex termed trypanosome lytic factor 2 (8–11). Both trypanosome lytic factor 1 and trypanosome lytic factor 2 protect against the African parasite *Trypanosoma brucei brucei* by inducing lysis of the parasite, and Hpr is suggested to play a vital role in this primate-specific defense mechanism (12, 13).

Recently we identified Hpr as a high affinity Hb-binding protein associating with Hb virtually as efficiently as Hp (14). Unlike Hp complex formation with Hb, Hpr complex formation with Hb does not increase Hb binding to CD163, thus indicating that some of the differences in the primary structure of Hp and Hpr are indispensable for high affinity receptor binding. In the present study, we have therefore designed and analyzed recombinant Hp/Hpr chimeric proteins as an approach to identify Hp regions/residues essential for the high affinity binding of Hp-Hb to CD163.

Experimental Procedures

**Construction of Plasmids for Expression of Recombinant Hp, Hpr, and Hp/Hpr Chimeras**—The plasmid constructs composed of human Hp1 wild type (WT) or human Hpr WT

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<sup>2</sup> The abbreviations used are: Hp, haptoglobin; Hb, hemoglobin; proHp, Hp precursor; Hpr, haptoglobin-related protein; WT, wild type; MS, mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization-MS; SPR, surface plasmon resonance; HPLC, high pressure liquid chromatography.

inserted into the KpnI and XhoI sites of the mammalian expression vector pcDNA5/FRT (Invitrogen, Taastrup, Denmark) were described recently (14). By means of site-directed mutagenesis, a large panel of plasmid constructs encoding Hp/Hpr chimeras was established. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene) using the pcDNA5/FRT-Hp1 WT construct as the initial template. Plasmid DNA was isolated by the Qiagen Maxiprep method (Qiagen, Albertslund, Denmark) and sequenced before transfection. The following constructs designated according to their encoded Hp and Hpr amino acid composition were designed: *chimera 1*, Hpr( $\alpha$ -chain)/Hp( $\beta$ -chain) (the chimeric protein consisting of the Hpr  $\alpha$ -chain and the Hp  $\beta$ -chain); *chimera 2*, Hp C33F (the Hp protein with a single mutation of Cys-33 to Phe); *chimera 3*, Hp( $\alpha$ -chain)/Hpr( $\beta$ -chain) (the chimeric protein consisting of the Hp  $\alpha$ -chain and the Hpr  $\beta$ -chain); *chimera 4*, Hpr( $\alpha$ -chain)/Hpr( $\beta$ -chain 104–234)/Hp( $\beta$ -chain 234–347) (the chimeric protein consisting of the Hpr  $\alpha$ -chain, Hpr  $\beta$ -chain residues 104–234, and Hp  $\beta$ -chain residues 234–347); *chimera 5*, Hpr( $\alpha$ -chain)/Hpr( $\beta$ -chain 104–253)/Hp( $\beta$ -chain 253–347) (the chimeric protein consisting of the Hpr  $\alpha$ -chain, Hpr  $\beta$ -chain residues 104–253, and Hp  $\beta$ -chain residues 253–347); *chimera 6*, Hpr( $\alpha$ -chain)/Hpr( $\beta$ -chain 104–265)/Hp( $\beta$ -chain 265–347) (the chimeric protein consisting of the Hpr  $\alpha$ -chain, Hpr  $\beta$ -chain residues 104–265, and Hp  $\beta$ -chain residues 265–347); *chimera 7*, Hpr( $\alpha$ -chain)/Hpr( $\beta$ -chain 104–265, C260V)/Hp( $\beta$ -chain 265–347) (the chimeric protein consisting of the Hpr  $\alpha$ -chain, Hpr  $\beta$ -chain residues 104–259 and 261–265, and Hp  $\beta$ -chain residues 259 and 265–347); *chimera 8*, Hp C33F, V259C (the Hp protein with mutations of Cys-33 to Phe and Val-259 to Cys); and *chimera 9*, Hp E261K, K262W, T264A (the Hp protein with mutations of Glu-261 to Lys, Lys-262 to Trp, and Thr-264 to Ala).

**Establishment of Cell Lines Stably Transfected with Hp/Hpr Chimeras**—Flp-In 293 cells (Invitrogen) were grown in Dulbecco's modified Eagle's medium (Cambrex Bioscience Verviers, Verviers, Belgium) supplemented with 10% fetal calf serum, 2 mM glutamine, and 100  $\mu$ g/ml zeocin (Invitrogen) and transfected using FuGENE 6 (Roche Diagnostics, Hvidovre, Denmark). Stable transfectants were selected with 150  $\mu$ g/ml hygromycin B (Invitrogen). For visualization of expression products, culture medium harvested from cells grown in serum-free 293 medium (Invitrogen) was subjected to SDS-PAGE and subsequent immunoblotting using a rabbit polyclonal anti-human Hp antibody (DAKOCytomation, Glostrup, Denmark). The generation of Flp-In 293 cells stably transfected with Hp WT and Hpr WT was described previously (14).

**Purification of Hp, Hpr, and Hp/Hpr Chimeras by Hb Affinity Chromatography**—Purification of Hp, Hpr, and Hp/Hpr chimeras was performed by subjecting the harvested serum-free 293 cell culture medium containing secreted expression products to Hb affinity chromatography, essentially as described (14, 15). Affinity columns were prepared by coupling 10 mg of Hb A<sub>0</sub> (Sigma, Brøndby, Denmark) to 1 g of CNBr-activated Sepharose 4B (Amersham Biosciences, Hørsholm, Denmark) essentially as described by the manufacturer. After allowing the expression products to bind the column material, the columns

were washed extensively with phosphate-buffered saline (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15 M NaCl, 0.6 mM CaCl<sub>2</sub>), pH 7.4, and bound proteins subsequently eluted by 5 M urea containing 0.15 M NaCl. The collected material was dialyzed overnight at 4 °C against phosphate-buffered saline, pH 7.4, with one buffer change and analyzed by SDS-PAGE followed by silver staining to visualize the purified protein products.

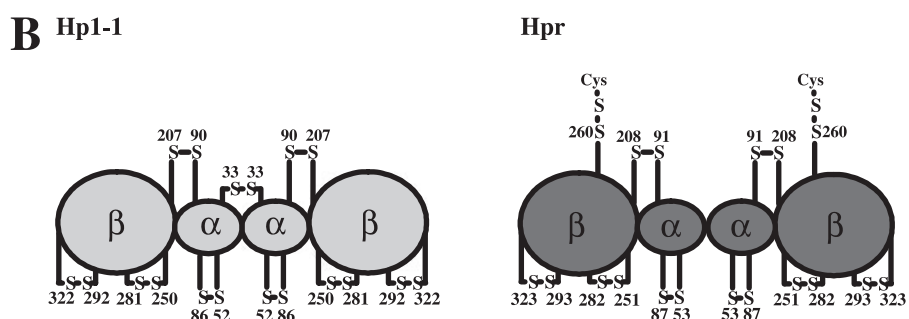
**Automated Edman Degradation**—Samples were analyzed by automated Edman degradation using an Applied Biosystems Procise 491HT sequencer with on-line phenylthiohydantoin HPLC analysis. The instruments were operated according to instructions by the manufacturer.

**Mass Spectrometry (MS)**—Approximately 40  $\mu$ g of recombinant Hpr in 30 mM HEPES, pH 8.3, containing 5 M guanidinium hydrochloride was *S*-carboxyamidomethylated by adding iodoacetamide to a final concentration of 10 mM. The reaction was allowed to proceed for 30 min before the material was acidified and desalted on to a 2.1  $\times$  220 mm Aquapore RP-300 C<sub>8</sub> reverse-phase HPLC column (Brownlee Labs) connected to an AKTA explorer (GE Healthcare). Bound proteins were eluted by a linear gradient from 0.1% trifluoroacetic acid (solvent A) to 90% acetonitrile, 0.08% trifluoroacetic acid (solvent B) (1% B min<sup>-1</sup>). The column was operated at 23 °C at a flow rate of 200  $\mu$ l min<sup>-1</sup>. Protein was detected at 220 and 280 nm, and fractions were collected manually. The fraction containing Hpr was lyophilized, redissolved in 50 mM Tris-HCl, 137 mM NaCl, pH 7.4, and digested by the addition of trypsin (sequence grade, Promega) at a 1:20 ratio for 16 h at 37 °C. The tryptic peptides were subsequently separated by reverse-phase HPLC as described above. Fractions were collected manually and analyzed by MALDI-MS before and after reduction using a Q-ToF Ultima Global mass spectrometer (Micromass/Waters Corp.) calibrated using a mixture of polyethylene glycol-200, -600, -1000, -2000, and NaI. The acquired mass spectra were mass-corrected using glu-fibrinopeptide B as an external calibrant. Aliquots of fractions selected for subdigestion using *Staphylococcus aureus* V8 protease (Sigma) were lyophilized and redissolved in 50 mM NH<sub>4</sub>HCO<sub>3</sub>. The digestions were allowed to proceed for 30 min at 37 °C. The generated peptides were recovered by using StageTips (C<sub>18</sub>, Proxeon) as recommended by the manufacturer and spotted onto a MALDI sample target using 1  $\mu$ l of matrix solution (0.4% (w/v) recrystallized  $\alpha$ -cyano-4-hydroxy-cinnamic acid in 70% (v/v) acetonitrile, 0.03% (v/v) trifluoroacetic acid) and analyzed by MALDI-MS as described above.

**Surface Plasmon Resonance (SPR) Analysis**—CD163 binding of Hb A<sub>0</sub>, Hp, Hpr, Hp/Hpr chimeras (all 25  $\mu$ g/ml) alone or complexes of Hb A<sub>0</sub> with Hp, Hpr, or Hp/Hpr chimeras was studied by SPR analysis on a Biacore 3000 instrument (Biacore, Uppsala, Sweden), essentially as described (2). Biacore sensor chips (type CM5) were activated with a 1:1 mixture of 0.2 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinimide in water according to instructions by the manufacturer. CD163 purified from human spleen (1) was immobilized in 10 mM sodium acetate, pH 4.0, and remaining binding sites were blocked with 1 M ethanolamine, pH 8.5. A control flow cell was made by performing the activation and blocking procedure only. The SPR signal generated from immobilized recombinant protein corresponded to 60–70 fmol

**A**

Hp1	1	MSALGAVIALLLWG-QLFAVD	SGNDVTDIAD	GGCPKPEIAHGYVEHSVR	49
Hpr	1	MSDLGAVISLLLLWGRQLFALY	SGNDVTDISDDR	FFPKPPEIANGYVEHLFR	50
Hp1	50	YQCKNYIKLRTEGDGVYTLN	NEKQWINKAVGDKLPECEAV	CGKPKNPANP	99
Hpr	51	YQCKNYIKLRTEGDGVYTLN	DKKQWINKAVGDKLPECEAV	CGKPKNPANP	100
		$\alpha \downarrow \beta$			
Hp1	100	VQRILGGHLDAGSFPWQAKMV	SHHNLTTGATLINEQWLLTT	AKNLFNLH	149
Hpr	101	VQRILGGHLDAGSFPWQAKMV	SHHNLTTGATLINEQWLLTT	AKNLFNLH	150
Hp1	150	SENATAKDIAPTLTLYVGKKQ	LVEIEKVVLPNYSQVDIGLIK	LKQKVS	199
Hpr	151	SENATAKDIAPTLTLYVGKKQ	LVEIEKVVLPNYSQVDIGLIK	LKQKVLV	200
Hp1	200	NERVMPICLPSKDYAEVGRV	GVYSGWGRNANFKFTDHLK	YVMLPVADQDQ	249
Hpr	201	NERVMPICLPSKNYAEVGRV	GVYSGWQSDNFKLTDHLK	YVMLPVADQYD	250
Hp1	250	CIIRHYEGSTVPEKKT	PKSPVGVQPILNEHTFC	AGMSKYQEDTCYGDAGSA	299
Hpr	251	CITHYEGSTCPKWK	AKSPVGVQPILNEHTFC	VGMSKYQEDTCYGDAGSA	300
Hp1	300	FAVHDLLEEDTWYATGILS	FDKSCAVAEYGVYVKVTSIQ	DWVQKTIEN	347
Hpr	301	FAVHDLLEEDTWYAAGILS	FDKSCAFAEYGVYVKVTSIQ	DWVQKTIEN	348



**FIGURE 1. Sequence alignment of Hp and Hpr and determination of disulfide bridges of human Hpr.** A, sequence alignment of human Hp1 (GenBank<sup>TM</sup> accession number X00637) and human Hpr (GenBank accession number NM\_020995.2). Amino acid residue differences between Hp and Hpr are shown in *black*, and the presence of cysteines is indicated by a *horizontal line*. The cleavage sites of the 18-residue Hp signal peptide and between the  $\alpha$ - and  $\beta$ -chains are indicated by *arrows*. B, schematic presentation of the Hp and Hpr molecules indicating the disulfide patterns. *Small and large spheres* represent the  $\alpha$ - and  $\beta$ -chains, respectively. The disulfide pattern of Hp is based on the study by Kurosky *et al.* (18), whereas the Hpr disulfide arrangement is defined on recombinant Hpr in the present study. Despite the missing inter- $\alpha$ -chain disulfide bridge in Hpr, a recent study (14) suggests a non-covalent linkage between Hpr ( $\alpha\beta$ )-monomers to form an ( $\alpha\beta$ )<sub>2</sub> structure (see "Discussion").

of receptor/mm<sup>2</sup>. Samples were dissolved in 10 mM Hepes, 150 mM NaCl, 2.0 mM CaCl<sub>2</sub>, 1.0 mM EGTA, and 0.005% Tween 20, pH 7.4. Sample and running buffers were identical. Regeneration of the sensor chip after each analysis cycle was performed with 10 mM glycine, pH 4.0, containing 20 mM EDTA and 500 mM NaCl. The Biacore response is expressed in relative response units, *i.e.* the difference in response between protein and control flow channel. Kinetic parameters were determined by BIAevaluation 4.1 software using a Langmuir 1:1 binding model.

The effect of the synthetic peptides RHYEGSTVPEKKTPKSPVGVQPILNEHT and THYEGSTVPKWKAPKSPVGVQPILNEHT (KJ ROSS-Petersen ApS, Klampenborg, Denmark) on the binding of Hp-Hb to immobilized CD163 was studied using an Hp (1-1)-Hb A<sub>0</sub> complex formed by mixing 8  $\mu$ g/ml Hb A<sub>0</sub> with 8  $\mu$ g/ml plasma-derived Hp1-1 (Sigma). The synthetic peptides were dissolved in sample buffer, and experiments were performed in a concentration range from 0 to 750  $\mu$ M of peptides. Sensorgrams were monitored, and the relative response at 600 s was used in calculation of the percentage of binding of the Hp-Hb complex.

### Modeling of the Hp Structure—

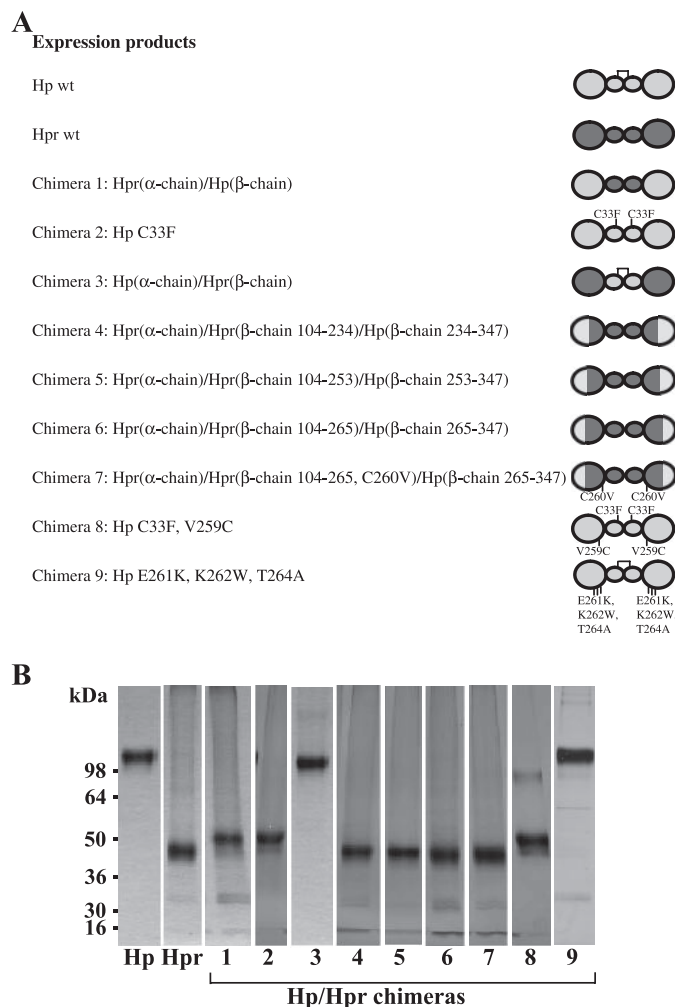
The core of the model of human Hp was constructed using the Swiss-Model server (16). SwissModel selected three structures of C1r (Protein Data Bank code: 1MD8, 1MD7, 1GPZ) and one structure of C1s (Protein Data Bank code: 1ELV) as templates for the modeling. Alternative suggestions for the structure of Hp loop 1 were identified by locating the nearly conserved sequence pattern PXXXXPXX-PXXXXP found in Hp in the set of non-redundant sequences of the Protein Data Bank using PATTERNSEARCHDB (Biology Workbench). Of 32 occurrences of the pattern, the loop from bovine mitochondrial aconitase (Protein Data Bank code: 1ACO) (residues 320–333) was chosen based on the agreement between endpoints of the loop and the core generated by Swiss-Model. The loop was incorporated into the Hp model using O (17).

## RESULTS

**Comparison of the Primary Structure of Hp and Hpr and Determination of the Disulfide Bridge Pattern of Hpr**—Fig. 1A shows an alignment of Hp1 and Hpr with the 27-amino-acid residue differences in the  $\alpha$ - and  $\beta$ -chains indicated in *black*. The differences include 2 cysteine residues, the Cys-260 in the Hpr  $\beta$ -chain, which is a replacement of a

Val in Hp, and Cys-33 in Hp, which is replaced by Phe in Hpr. The latter difference abolishes the covalent stabilization of the structure because Cys-33 mediates the disulfide bridging of the  $\alpha$ -chains of Hp (18). To investigate whether Hpr Cys-260 perturbs the disulfide bond pattern relatively to the disulfide arrangement known from Hp (18), purified recombinant Hpr was digested by trypsin, and the generated peptides were subsequently separated by reverse-phase HPLC. All fractions were analyzed by MALDI-MS, and selected fractions containing disulfide-linked peptides were subsequently subdigested using *S. aureus* V8 protease. This analysis revealed that the disulfide bridge pattern of Hpr was homologous to that of the Hp ( $\alpha\beta$ )-unit (Fig. 1B). Furthermore, the analysis showed that Cys-260 was cysteinylated. The latter conclusion was based on the finding that under non-reducing conditions, the peptide encompassing Cys-260 had a mass increase of 119 Da corresponding to the cysteinylation of the cysteine residue. The identification of Cys-260 as the cysteinylated residue was determined by enzymatic subdigestion of the disulfide-linked tryptic peptide. As Hpr contains an uneven number of cysteine residues, the inability to detect any





**FIGURE 2. Expression of recombinant Hp, Hpr, and Hp/Hpr chimeras.** A, overview of the recombinant Hp, Hpr, and Hp/Hpr chimeric proteins produced in the human 293 cell expression system. The overall structure of the expression products is shown schematically to the right. For clarity, all disulfide bridges, except the inter- $\alpha$ -chain disulfide bridge, have been omitted. B, silver-stained 8–16% SDS-polyacrylamide gel of the purified recombinant Hp, Hpr, and Hp/Hpr chimeric expression products. Expression products were purified by subjecting harvested culture medium to Hb affinity chromatography. Positions of the molecular size standard markers are indicated.

S-carboxyamidomethylated Cys-containing peptides further supports the presence of a cysteinylated.

**Expression of Recombinant Hp/Hpr Chimeric Proteins**—A site-directed mutagenesis strategy was based on the 27 amino acid residues, which are different in Hpr as compared with Hp. Fig. 2 shows the panel of Hp/Hpr chimeric proteins expressed and analyzed to determine the elements important for receptor recognition.

In agreement with Hp Cys-33 being responsible for the disulfide-mediated dimerization of ( $\alpha\beta$ )-monomers to form the Hp ( $\alpha\beta$ )<sub>2</sub> molecule (18), all expression products containing the C33F mutation primarily formed ( $\alpha\beta$ )-monomers of approximately half the size of the recombinant Hp WT ( $\alpha\beta$ )<sub>2</sub> molecule as determined by non-reducing SDS-PAGE. The mobility in SDS-PAGE was also influenced by the Hp S184H mutation disrupting a glycosylation motif of the type Asn-X-Ser/Thr. Expression products carrying this amino acid substitution

migrated slightly faster than expression products without the mutation.

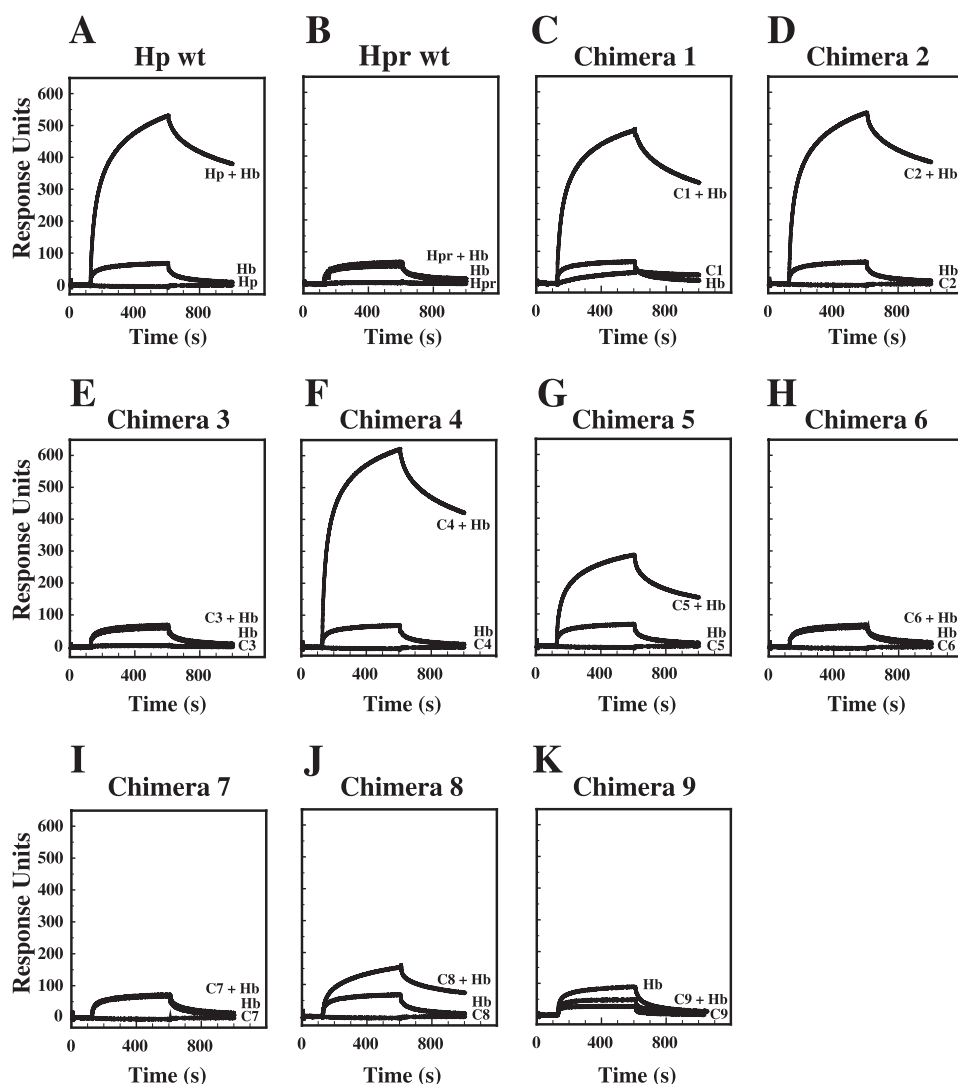
In accordance with recent data showing that only a low degree of cleavage of recombinant proHp to the  $\alpha$ - and  $\beta$ -chains takes place in 293 cells (19), amino-terminal sequencing showed that recombinant Hp remained largely uncleaved (14). However, the recombinant Hp bound to Hb has similar receptor binding activity as Hb in complex with native plasma-derived Hp (14). Surprisingly, the recombinant Hpr expressed in the 293 cells was fully cleaved into the  $\alpha$ - and  $\beta$ -chains (14), thus suggesting that it is accessible for cleavage by an alternative protease. However, in contrast to the native Hpr found in plasma (9, 10), recombinant Hpr was found to lack the hydrophobic signal peptide (14).

**Identification of the Hp  $\beta$ -Chain Region Involved in High Affinity Binding to CD163**—As expected, the panel of different Hp/Hpr chimeric expression products all bound Hb as evident by Hb affinity chromatography (Fig. 2B). Using SPR analysis, we subsequently examined the CD163 binding capacity of the purified proteins in complex with Hb (Fig. 3, Table 1). Replacement of the Hp  $\alpha$ -chain with that of Hpr had no significant effect on the CD163 binding as seen by comparison of Hp WT and the Hpr( $\alpha$ -chain)/Hp( $\beta$ -chain) chimera (Fig. 3, A and C). Concordantly, the Hp C33F mutant selectively missing the inter- $\alpha$ -chain disulfide bridge showed high affinity receptor binding (Fig. 3D). Thus, the residues specific for the Hpr  $\alpha$ -chain, including the C33F substitution, which prevents the disulfide-mediated ( $\alpha\beta$ )-dimer formation, do not affect the binding to CD163.

In striking contrast, substitution of the Hp  $\beta$ -chain with that of Hpr completely abolished the ability to increase Hb binding to CD163 as revealed by binding experiments using the Hp( $\alpha$ -chain)/Hpr( $\beta$ -chain) chimeric protein (Fig. 3E). In the next step, we analyzed mutant proteins consisting of the Hpr  $\alpha$ -chain and chimeric  $\beta$ -chain Hp/Hpr. Substituting the Hp residues 1–233 by the corresponding Hpr residues (1–234) had no significant impact on receptor binding (Fig. 3F). However, introducing the mutations corresponding to the Hpr residues 235–253 and 235–265 had a weak and completely abrogating effect, respectively, on the Hp-inducible increase in Hb binding to CD163 (Fig. 3, G and H). In essence, the triple substitution D248Y, Q249D, and R252T caused partial inhibition of high affinity receptor binding, and combining with the V259C, E261K, K262W, and T264A substitutions entirely eliminated high affinity receptor binding.

Introduction of the V259C mutation in Hp (corresponding to Cys-260 of Hpr) appeared interesting because Hpr Cys-260 is cysteinylated (Fig. 1B). However, reintroduction of the original Hp Val at this position failed to rescue high affinity binding (Fig. 3, H and I). On the other hand, introduction of a Cys at Hp position 259, even in the absence of the D248Y, Q249D, R252T, E261K, K262W, and T264A substitutions, caused a substantial decrease in receptor binding as seen by comparing the Hp C33F and Hp C33F, V259C mutant proteins (Fig. 3, D versus J).

Finally, the effect of the E261K, K262W, and T264A substitutions on receptor binding was tested in the absence of other residue alterations. Strikingly, as presented in Fig. 3, introduc-



**FIGURE 3. SPR analyses of binding of complexes of Hb and purified recombinant Hp, Hpr, or Hp/Hpr chimeras to CD163.** Purified Hb alone, purified recombinant Hp, Hpr, Hp/Hpr chimera 1–9 (C1–9) alone, or the complexes of Hb with Hp, Hpr, and Hp/Hpr chimeras were analyzed for binding to CD163 immobilized on a Biacore sensor chip. The recombinant protein analyzed is indicated above each panel (for designations of the chimeric proteins, refer to Fig. 2). Hb alone displayed low binding to CD163, whereas no binding of Hp, Hpr, or the Hp/Hpr chimeras alone to CD163 could be detected.

**TABLE 1**

**Kinetic parameters derived from SPR analysis of binding of Hb alone or complexes of Hb and purified recombinant Hp, Hpr, and Hp/Hpr chimeras to immobilized CD163**

The kinetic parameters were determined under the assumption that Hb alone binds CD163 as a dimer consisting of two globin subunits and that Hpr and Hp/Hpr chimeras harboring the Hp C33F substitution non-covalently dimerize to form the  $(\alpha\beta)_2$  structure (see discussion). The kinetic parameters were not determined in the cases of CD163-binding curve characteristics indistinguishable from those of Hb alone.  $K_d$ , dissociation constant;  $k_a$ , association rate constant;  $k_d$ , dissociation rate constant; ND, not determined.

Ligand	$K_d$	$k_a$	$k_d$
	M	1/MS	1/s
Hb	$3.7 \times 10^{-7}$	$7.6 \times 10^3$	$2.8 \times 10^{-3}$
Hp wt-Hb	$1.2 \times 10^{-8}$	$4.4 \times 10^4$	$5.3 \times 10^{-4}$
Hpr wt-Hb	ND	ND	ND
Chimera 1-Hb	$1.6 \times 10^{-8}$	$4.8 \times 10^4$	$7.6 \times 10^{-4}$
Chimera 2-Hb	$1.1 \times 10^{-8}$	$5.0 \times 10^4$	$5.5 \times 10^{-4}$
Chimera 3-Hb	ND	ND	ND
Chimera 4-Hb	$1.3 \times 10^{-8}$	$5.3 \times 10^4$	$7.1 \times 10^{-4}$
Chimera 5-Hb	$2.7 \times 10^{-8}$	$3.8 \times 10^4$	$1.0 \times 10^{-3}$
Chimera 6-Hb	ND	ND	ND
Chimera 7-Hb	ND	ND	ND
Chimera 8-Hb	$4.7 \times 10^{-8}$	$2.4 \times 10^4$	$1.1 \times 10^{-3}$
Chimera 9-Hb	ND	ND	ND

tion of these 3 amino acid residue substitutions in Hp was sufficient to obstruct high affinity receptor binding (compare A and K). Taken together, the binding analyses demonstrate that the region encompassing residues 234–264 of the Hp  $\beta$ -chain contains residues that are critical for receptor binding, in particular the residues Val-259, Glu-261, Lys-262, and Thr-264, which in Hpr are replaced by Cys, Lys, Trp, and Ala, respectively.

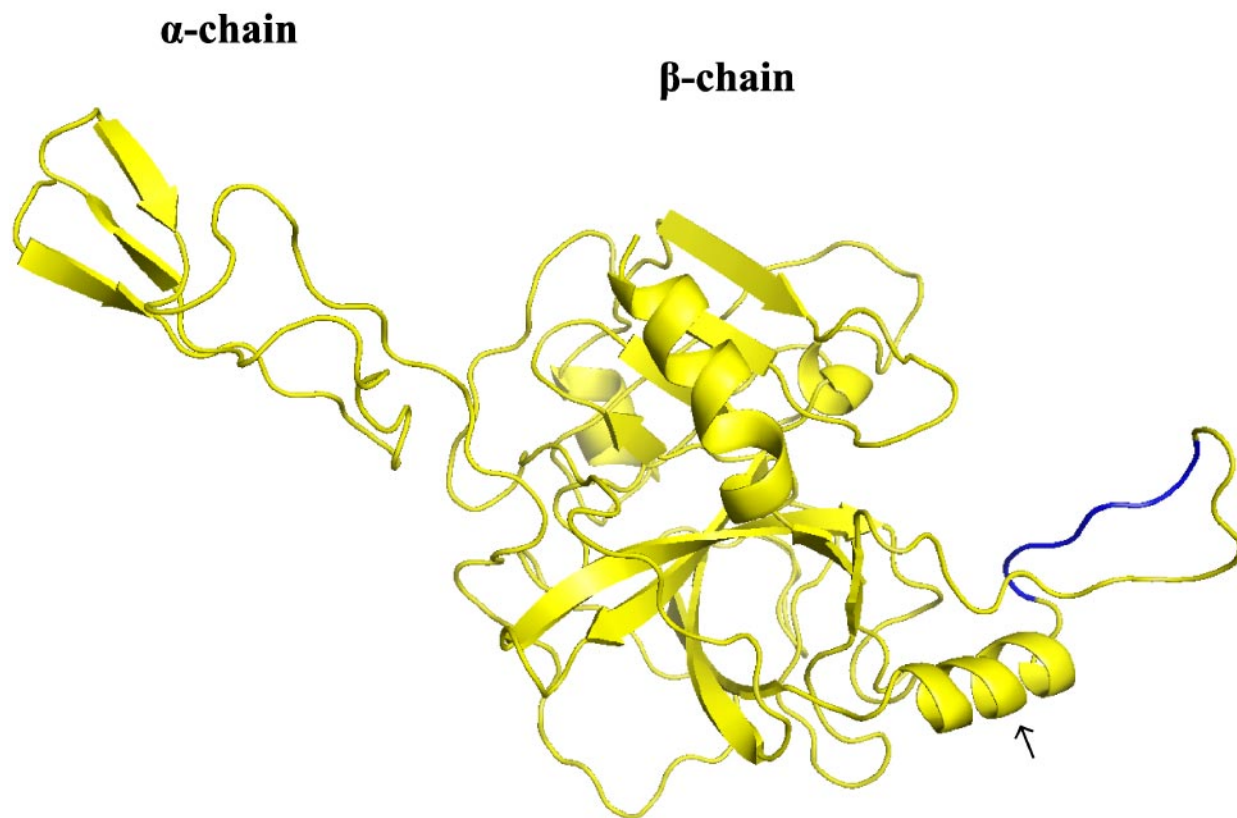
The determination of the structural fold of the complement control repeat and serine protease domains of complement components C1r and C1s by x-ray crystallography allows modeling of Hp as depicted in Fig. 4A. Due to the lack of similarity between the templates and the amino-terminal part of Hp, the model only comprises residues 43–346. Interestingly, the region identified as important for receptor binding represents a part of a loop, which has been designated as loop 1 of serine proteases (20). This is a unique part of Hp and Hpr as compared with serine proteases, which all have a considerably shorter loop (Fig. 4B). Due to the length of the loop and the lack of a known template structure, a reliable model for the loop structure cannot be obtained. The presence of 4 proline residues in the loop suggests a limited flexibility.

#### Inhibition of CD163 Binding of Hp-Hb by a Peptide Encompassing

**Hp Residues 252–279**—The role of the Hp loop 1 in receptor binding was also examined directly by analysis of the binding properties of a synthetic peptide corresponding to Hp residues 252–279. These data showed that the peptide, albeit with low affinity, efficiently inhibited Hp-Hb binding to CD163 (Fig. 5). In contrast, a peptide representing the corresponding loop of Hpr (except for Cys-260) was severalfold less efficient in inhibiting Hp-Hb binding to CD163 (Fig. 5).

#### DISCUSSION

In the present study, we have taken advantage of the homology between Hp and Hpr to design and analyze a panel of Hp/Hpr chimeric proteins. On this basis, it was possible to identify an Hp  $\beta$ -chain site essential for CD163 recognition of the Hp-Hb complex. The differences in 4 closely spaced amino acid residues (corresponding to Hp residues 259, 261, 262, and 264) explain that Hpr is unable to increase the low binding of Hb alone to CD163. The identified CD163-binding site repre-

**A****B**

<b>Hp</b>	222	VSGWGR-----NANFKFTDHLKYVMLPVADQDQCI	RHYEGST <b>VPEKKT</b> PKSPVGVQPILNEHTFCAGMSKYQ--	288
<b>Hpr</b>	223	VSGWGQ-----SDNFKLTDHLKYVMLPVADQYDCI	THYEGSTCPKWKAPKSPVGVQPILNEHTFCVGM	289
<b>C1s</b>	567	ISGWGR-----TEKRDRAVRLKAARLPVAPLRKCKE	VKVEKPTADAEAY-----VFTPNMICAGG-EKG--	624
<b>C1r</b>	593	VSGFGV-----MEEK-IAHDLRFVRLPVANPQACEN	WLRGKNRMD-----VFSQNMFCAGHPSLK--	646
<b>Trypsin</b>	141	ISGWGN-----TASSGADYPDELQCLDAPVLSQAK	CEASYPGK-----ITSNMF	191
<b>Chymotryp</b>	156	TTGWGK-----TKYNANKTPDKLQQAALPLLSNAE	CKKSWGRR-----ITDVMICAGASG---	205
<b>Thrombin</b>	501	VTGWGNLKETWTANVGKGQPSVLQVVNLPIVERPV	CKDSTRIR-----ITDNMFCAGYKPDEGK	559
<b>Factor X</b>	366	VSGFG-----RTHEKGRQSTRLKMLEVPYVDRNS	CKLSSSFI-----ITQNMFCAGYDT---K	410
<b>Hp</b>	289	-EDTCYGDAGSAFAVHDL	E-EDTWYATGILSFD-KSCAVA-EYGVYVKVTSIQD	347
<b>Hpr</b>	290	-EDTCYGDAGSAFAVHDL	E-EDTWYAAGILSFD-KSCAVA-EYGVYVKVTSIQD	348
<b>C1s</b>	625	-MDSCKGDSGGAFVQDPNDKTKFYAAGLVSWG-PQ	CG--TYGLYTRVKNYVDWIMKTMQENSTPRED---	688
<b>C1r</b>	647	-QDACQGDSSGGVFAVRDPN-TDRWVATGIVSWG-IG	CSR--GYGFYTKVLNYVDWIKKEMEE-----ED---	705
<b>Trypsin</b>	192	GKDSQCQGDSSGGPVVCN-----GQLQGVVSWG-D	GCAQKNKPGVYTKVYNYVKWIKNTIAANS-----	247
<b>Chymotryp</b>	206	-VSSCMGDSGGPLVCQ--K-DGAWTLVGIVSWGSD	TCSTSSPGVYARVTKLIPWVQKILAN-----	263
<b>Thrombin</b>	560	RGDACEGDSGGPFVVMKSPF--NNRWYQMGIVSWG-	EGCDRDGKYGFYTHVFLKKWIQKVIDQFGE-----	622
<b>Factor X</b>	411	QEDACQGDSSGGPHVTR--F-KDTYFVTGIVSWG-	EGCARKGKYGIYTKVTAFLKWIIDRSMKTRGLPKAK	483

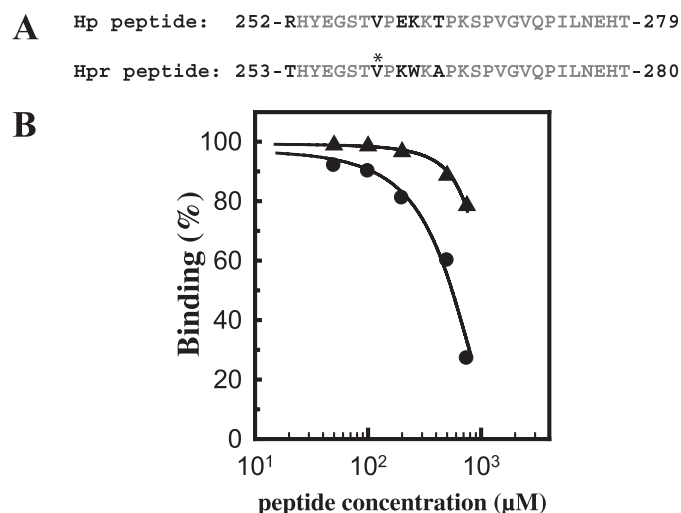
**FIGURE 4. Computer-modeled three-dimensional structure of Hp showing the relative orientation of the CD163-binding loop.** A, prediction of the three-dimensional structure of Hp based on the homology with complement components C1r and C1s, which are some of the closest homologues of Hp. The VPEKKT motif is indicated in blue. The arrow points to an  $\alpha$ -helical segment reported to be involved in Hb binding (23). B, multiple sequence alignment of serine protease domain-containing proteins, including Hp (GenBank accession number X00637), Hpr (GenBank accession number NM\_020995.2), complement component C1s (C1s, GenBank accession number X06596), complement component C1r (C1r, GenBank accession number X04701), trypsin (GenBank accession number M22612), chymotrypsin (chymotrp, GenBank accession number M24400), thrombin (GenBank accession number M17262), and coagulation factor X (factor X, GenBank accession number K03194). Loop 1 is shown in black, and the VPEKKT motif of Hp is highlighted in blue.

sents an extension of loop 1 of the serine protease domain. This loop and the missing catalytic triad are the most striking differences between Hp/Hpr and archetype serine proteases. Interestingly, loop 1 of serine proteases is known to determine the so-called S1 specificity, which is the crucial element of substrate selectivity in these enzymes (20, 21).

Other amino acid differences in Hpr and Hp, including that affecting disulfide-bridging of the  $\alpha$ -chains in Hp, had little or no effect on CD163 binding.

Electron microscopic studies suggest that the Hb dimer binds the Hp  $\beta$ -chain 127° off the Hp  $\alpha\beta$  axis (22). Moreover, by using a series of truncated Hp proteins, residues 182–222





**FIGURE 5. Inhibition of Hp-Hb binding to CD163 by a peptide encompassing Hp residues 252–279.** *A*, peptides with the sequence of the Hp and Hpr loop 1. The sequences represent residues 252–279 of Hp and residues 253–280 of Hpr (except for Cys-260, which is substituted by the corresponding Val of Hp, denoted with an asterisk, to avoid inexpedient disulfide formation). *B*, inhibitory effects of the Hp and Hpr-like peptides on Hp-Hb binding to CD163 as assayed by SPR analysis. Plasma-derived human Hp (1-1) in complex with Hb was tested for binding to immobilized CD163 in the presence of increasing concentrations of the Hp peptide (circles) and Hpr-like peptide (triangles) indicated in *A*. The effect of the peptides on binding of Hp-Hb to CD163 is indicated relative to Hp-Hb binding to CD163 in the absence of peptide.

and 243–263 of Hp1 have been reported to confer Hb binding (23). The latter segment includes an  $\alpha$ -helix positioned amino-terminally to loop 1 (Fig. 4A) as well as the amino-terminal part of loop 1 itself and is thus positioned closely to, and partly overlapping with, the herein identified CD163-binding site around Hp residues 259–264. As CD163 only binds Hp when complexed to Hb, the Hb- and CD163-binding sites in Hp cannot overlap. Accordingly, our data indicate that Hp residues 259–264 are not part of a site for direct binding of Hb.

It remains yet unknown how the loop 1 structure and position are affected by the binding of Hb to Hp. The binding of Hb to Hp is one of the strongest plasma protein interactions reported, indicating a large contact area that may cause substantial conformational change of the Hb and Hp structures upon complex formation. The present model shows that the Hp loop 1 is surface-exposed, but it is possible that its positioning is more favorable for CD163 recognition after Hb is bound to Hp. Furthermore, the loop may only be a part of a non-linear structure that binds CD163, and this may, at least partly, explain the finding that the loop-derived Hp peptide only inhibits receptor binding with low affinity. A non-linear CD163-binding structure in the Hp-Hb complex may also include a part of Hb in accordance with the juxtaposition of the Hb- and CD163-binding sites in Hp (Fig. 4A). This possibility does have some support in a recent report on low affinity binding of Hb to CD163 (24). We can confirm a low affinity binding of Hb to CD163 (apparent  $K_d = 0.4 \mu\text{M}$  for Hb versus apparent  $K_d = 12 \text{ nM}$  for Hp-Hb) as shown here in the SPR data (Fig. 3, Table 1). An exact comparison of the affinity of the two ligands is difficult because of the oligo-

meric nature of the Hp-Hb complex. However, in agreement with a large difference in affinity, we find in CD163-transfected cells that Hb is an at least 100-fold weaker inhibitor of  $^{125}\text{I}$ -Hp-Hb uptake than Hp-Hb.<sup>3</sup>

Our data do not indicate any direct engagement of the Hp  $\alpha$ -chain in receptor binding. Instead, the main role of the  $\alpha$ -chain seems to be to assemble Hp molecules, leading to gain in functional receptor affinity. One or two disulfide bridges (dependent on Hp phenotype) stabilize this  $\alpha$ -chain- $\alpha$ -chain interaction in Hp. The inter- $\alpha$ -chain disulfide bridge is missing in Hpr. Nevertheless, Hpr seems to have a size corresponding to that of the Hp1-1 molecule (14), probably because of non-covalent forces between the  $\alpha$ -chains (22). Such a non-covalent dimerization may explain the present finding that exchange of the Hp  $\alpha$ -chain with that of Hpr has no measurable effect on CD163 binding.

In conclusion, the present study has allocated a CD163-binding site on Hp within Hp-Hb complexes to a presumed external loop, positioned in a peripheral and, apparently, rather accessible region of the Hp  $\beta$ -chain. Hopefully, the future may define the crystallographic structure of the complex of Hp-Hb-CD163 to determine this intriguing interplay between the Hb units, a serine protease domain, and the CD163 scavenger receptor cysteine-rich repeats.

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**A Unique Loop Extension in the Serine Protease Domain of Haptoglobin Is Essential for CD163 Recognition of the Haptoglobin-Hemoglobin Complex**  
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