Haptoglobin-related protein is a high-affinity hemoglobin-binding plasma protein

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Contributions:
MJN has performed research (major part) and written the paper; SVP has performed research (amino-terminal sequencing, MALDI mass spectrometry analysis); CJ has performed research (SPR analysis); CO has performed research (amino acid analysis); DR has studied the sickle cell patients; HJM has performed research (gel filtration analysis, serum haptoglobin measurements); SKM has designed the study and written the paper.

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Abstract

Haptoglobin-related protein (Hpr) is a primate-specific plasma protein associated with apolipoprotein L-I (apoL-I)-containing high density lipoprotein (HDL) particles shown to be a part of the innate immune defense. Despite the assumption hitherto that Hpr does not bind to hemoglobin, the present study revealed that recombinant Hpr binds hemoglobin as efficiently as haptoglobin (Hp). However, in contrast to Hp, Hpr did not promote any high-affinity binding to the scavenger receptor CD163. Binding of hemoglobin to circulating native Hpr incorporated into the HDL fraction was indicated by hemoglobin-affinity precipitation of plasma Hpr together with apoL-I. In conclusion, plasma has two high-affinity hemoglobin-binding haptoglobins instead of one, but only Hp-hemoglobin complexes are efficiently recognized by CD163. Circulating Hpr-bound hemoglobin should therefore be taken into consideration when measuring ‘free’ plasma hemoglobin. Furthermore, Hpr-bound hemoglobin might contribute to the biological activity of the circulating apoL-I/Hpr-containing HDL particles.
Introduction

The primate-specific Hpr is a plasma protein with 91% sequence identity to the 1-1 phenotype of Hp, the hemoglobin-binding protein in plasma. Hpr is a core component of a subclass of HDL referred to as trypanosome lytic factor-1 (TLF1) based on its ability to induce lysis of the African parasite trypanosoma T. b. brucei, which primarily infects non-primate mammals.\(^1\,^2\) Aside from Hpr, the main TLF1 protein components are apolipoprotein A-I (apoA-I)\(^3\,^4\) and the primate-specific apoL-I.\(^5\) Both Hpr and apoL-I have been reported to possess lytic activity.\(^2\,^3\,^5\,-^7\)

Like Hp, Hpr is synthesized as an ~45 kDa protein that is proteolytically cleaved to form an α- and a β-chain which remain connected via a disulfide bond. However, in contrast to Hp, Hpr lacks a glycosylation site and a cysteine involved in inter-α-chain bonding.\(^8\) Human Hp has either one (Hp1 genotype) or two (Hp2 genotype) of such cysteines leading to the formation of the covalent \((\alpha\beta)_2\) structure (Hp1-1 phenotype) or larger \((\alpha\beta)\)–oligomers (Hp2-2 and Hp2-1 phenotypes).\(^9\) Furthermore, the α-chain of Hpr differs from that of Hp by containing the hydrophobic signal peptide,\(^2\,^4\) which may explain the association of Hpr to lipoprotein particles.

Hp avidly binds hemoglobin released into plasma during physiological and pathological hemolysis thereby preventing heme-mediated oxidative side effects in the kidney and other organs.\(^10\,^11\) Formation of the Hp-hemoglobin complex promotes high affinity binding to the monocyte/macrophage-specific scavenger receptor CD163 which mediates internalization of the complex.\(^12\) In contrast, immunoprecipitation experiments with TLF1 have suggested that Hpr is inactive in terms of hemoglobin binding.\(^13\,^14\) Due to the high degree of sequence similarity between Hp and Hpr we have investigated the hemoglobin- and receptor-binding potential of Hpr using a direct molecular approach.
Study design

Expression and purification of recombinant Hp1-1 and Hpr

Hp1 cDNA was amplified from a human foetal liver cDNA library (Clontech, Palo Alto, CA) and ligated into the pcDNA5/FRT vector (Invitrogen, Taastrup, Denmark). A construct encoding Hpr was generated by multiple rounds of site-directed mutagenesis (Quick change, Invitrogen). Flp-In 293 cells (Invitrogen) were transfected using FuGENE 6 (Roche Diagnostics, Hvidovre, Denmark) and stable transfectants selected using 150 µg/ml hygromycin B (Invitrogen). Serum-free 293 SFM II medium (Invitrogen) was collected from expression cultures and subjected to hemoglobin A0 (Sigma, Brøndby, Denmark)-affinity chromatography essentially as described. For amino-terminal sequencing, the purified proteins were heated in SDS-PAGE sample buffer for 3 min at 80 °C, separated by SDS-gel electrophoresis, and subsequently transferred to a polyvinylidene difluoride membrane as described. Automated Edman degradation was performed by using an Applied Biosystems Model 477A sequencer with on-line phenylthiohydantoin analysis by HPLC (Applied Biosystems Model 120A). In agreement with recent data demonstrating a very low degree of cleavage of recombinant Hp to α- and β-chains in 293 cells, amino-terminal sequencing of recombinant Hp yielded only the sequence of the mature α-chain amino-terminus (VDSGNDVTDIAD). Two amino-terminal sequences in equimolar amounts were obtained for recombinant Hpr (LYSGNDVTDISD and ILGGHLDALKSF).

Surface Plasmon Resonance (SPR) analysis

SPR analysis was performed on a Biacore 3000 instrument (Biacore, Sweden) as described. Hemoglobin A0 (Sigma) was immobilized at a concentration of 10 µg/ml in 10 mM sodium acetate pH 6.0. The resulting densities were 50-60 fmol/mm² (experiments in Fig. 1B) or 70-80 fmol/mm².
(experiments in Fig. 1C). Sample and running buffer was 10 mM Hepes, 150 mM NaCl, 3.0 mM CaCl₂, 1.0 mM EGTA, and 0.005 % Tween-20, pH 7.4.

**Hemoglobin-precipitation of human plasma proteins**

Random plasma and serum samples with normal Hp levels (>0.6 g/L) from the routine laboratory of the Department of Clinical Biochemistry, Aarhus Hospital or from steady-state patients with sickle cell anemia (HbSS) (Hp concentration <0.1 g/L) were incubated with hemoglobin-coupled Sepharose, bovine serum albumin (BSA)-coupled Sepharose, or underivatized Sepharose. Bound proteins were analyzed by immunoblotting (using rabbit polyclonal anti-human Hp antibody (DAKOCytomation, Glostrup, Denmark)) and amino-terminal sequencing/mass spectrometry essentially as described. The concentration of Hp was determined by ELISA using biotinylated anti-human Hp (DAKOCytomation).

**Quantification of Hpr levels**

Immunostained Hpr was visualized by using the ECL Western blot detection system (Amersham Biosciences, Hillerød, Denmark) and a FUJIFILM LAS-1000 luminescence image analyzer. Quantification relatively to standards of recombinant Hpr was performed by using the MultiGauge 3.0 software. The concentration of recombinant Hpr was determined by protein hydrolysis and amino acid analysis.
Results and discussion

Hpr and Hp (1-1 phenotype) were expressed as recombinant proteins in a mammalian 293 cell expression system. Binding of hemoglobin to Hpr was indicated by the surprising observation that hemoglobin-affinity chromatography was applicable for purification of both expression products. In agreement with the findings on the authentic proteins, non-reducing denaturing gel electrophoresis of the recombinant proteins showed migration of Hpr and Hp corresponding to ∼45 kDa and ∼110 kDa proteins, respectively (Fig. 1A). However, non-denaturing S-200 gel filtration showed identical elution of Hpr and Hp1-1 (data not shown), indicating that Hpr non-covalently also associates into a dimeric (αβ)2 structure.

SPR analysis showed an almost irreversible and virtually indistinguishable high affinity binding of hemoglobin A0 to both recombinant proteins (Fig. 1B). Similar SPR data were obtained for hemoglobin A0, hemoglobin A2, and hemoglobin S (data not shown). Hpr efficiently competed for binding of Hp to hemoglobin and vice versa (Fig. 1C). The signal peptide of the recombinant Hpr α-chain was found to be removed in contrast to that of plasma-derived Hpr. However, since hemoglobin is known to bind to the β-chain of Hp it seems unlikely that this difference affects hemoglobin binding to Hpr.

In contrast to the similar hemoglobin-binding properties, Hpr and Hp in complex with hemoglobin showed a significant difference in binding to CD163 (Fig. 1D). Whereas the Hp-hemoglobin complex formation induced a considerable increase in binding to CD163 relative to hemoglobin alone, such effect was not apparent for the Hpr-hemoglobin complex (Fig. 1D). Accordingly, SPR analysis showed no interference of Hpr with Hp-hemoglobin binding to CD163 (data not shown).

To show hemoglobin-binding activity of native Hpr in plasma, we identified human plasma proteins precipitated by hemoglobin-Sepharose. Immunoblotting and mass spectrometry/ami-
terminal sequencing (Fig. 2) identified Hp in multiple sizes and Hpr (containing the signal peptide) in a 45 kDa monomeric and a 90 kDa form. The 90 kDa form is in agreement with previous data reporting an SDS-resistant dimeric form of Hpr. Reducing SDS-PAGE and subsequent immunoblotting of hemoglobin affinity-purified proteins from the Hp2-1 serum displayed in Fig. 2A showed the characteristic difference in migration of the α-subunits specific for Hp1, Hp2 and Hpr (Fig. 2B). The staining intensities of these bands comply with the measured plasma concentration (see below) of Hpr amounting to ~1/10-1/20 of the Hp concentration. ApoL-I (Fig. 2C) and apoA-I (not shown) were also identified as hemoglobin-precipitated proteins suggesting that Hpr bound to hemoglobin-Sepharose was associated with TLF1 particles.

By quantitative Western blotting using recombinant Hpr as standard, we measured the Hpr monomer levels in 18 individuals with normal Hp levels (>0.6 g/L) to 40.7±14.3 mg/L and in 13 patients with low Hp levels due to sickle cell anemia and extensive intravascular hemolysis (Hp concentration <0.1 g/L) to 49.9±22.5 mg/L. Thus, in contrast to Hp the plasma concentration of Hpr appears to be unaffected by hemolysis. The depletion of Hp but not Hpr upon hemolysis was further confirmed by Western blot analysis (data not shown) of a large set (n= 15) of anonymous routine samples with low Hp levels and biochemical parameters indicating intravascular hemolysis.

The present data explains the previous observation that two patients with severe hemolysis, due to paroxysmal nocturnal hemoglobinuria, had depletion of ~98% of the Hp pool in plasma but virtually normal levels of Hpr. However, this observation is likely not due to lack of binding of Hpr to hemoglobin as originally suggested, but may be a consequence of the pronounced difference in CD163 binding of Hpr-hemoglobin and Hp-hemoglobin. Furthermore, our data suggest that the hemoglobin previously reported to be present in TLF1 particles is not a contaminant but represents Hpr-bound hemoglobin.
The absent induction of high affinity binding to CD163 when hemoglobin binds to Hpr indicates that some of the differences in the primary structure of Hp versus Hpr are essential for receptor binding. Furthermore, the very low affinity for CD163 and the association with high molecular weight particles resisting renal clearance, suggest an extended circulation time of Hpr-bound hemoglobin relative to free and Hp-associated hemoglobin. Although a previous study\textsuperscript{13} of hemoglobin-exposed TLF\textsubscript{1} particles did not reveal increased trypanosoma-lytic activity, it is intriguing to speculate that the oxidative potential of Hpr-bound hemoglobin may be a part of the innate immune defense as originally proposed.\textsuperscript{2} Moreover, it could be speculated that association of Hpr-bound hemoglobin with the anti-oxidant apoA-I\textsuperscript{24,25} in plasma may protect the circulatory system from the oxidative properties of hemoglobin which may be uncovered subsequent to internalization by \textit{T. b. brucei}.

Non-immune biological activity of circulating hemoglobin bound to HDL particles should also be considered. Hemoglobin released into plasma or added to plasma as a blood substitute scavenges NO, and this is associated with smooth muscle dystonias, endothelial dysfunction, and thrombosis.\textsuperscript{26,27} It will be highly important to investigate whether this toxicity of hemoglobin is influenced by circulating plasma Hpr/HDL.

Finally, the presence of two hemoglobin-binding haptoglobins in plasma instead of one has to be taken into account when measuring haptoglobin and ‘free’ hemoglobin in plasma, particularly in patients with low Hp levels where Hpr becomes a prominent hemoglobin-binding protein.

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References


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Figure legends

Fig. 1. Identification of recombinant Hpr as a hemoglobin-binding protein by hemoglobin-affinity chromatography and SPR analyses. A, Silver-stained SDS-polyacrylamide gels of hemoglobin affinity-purified recombinant human Hp (phenotype 1-1, rHp1-1) and Hpr (rHpr). B, SPR analysis of the binding of a range of concentrations of rHp1-1 and rHpr to immobilized hemoglobin A0 (Kd calculated to 2.3 nM and 13 nM, respectively. The slow dissociation (arrows, $k_d < 2 \times 10^{-4} \text{ s}^{-1}$) indicates an almost irreversible binding. The concentration of rHp1-1 and rHpr is shown to the right of each curve. C, SPR analysis of binding of rHpr or rHp1-1 to hemoglobin A0 in the presence of rHp1-1 and rHpr, respectively. The curves (solid lines) show binding of rHp1-1 (left panel) or rHpr (right panel) to the hemoglobin chip and subsequent binding of rHpr (arrow, left panel) and rHp1-1 (arrow, right panel). The dashed lines are transposed curves (from an identical chip) showing binding to the chip without prebound rHp1-1/rHpr, i.e. the expected binding in the absence of competitive inhibitor. D, SPR analysis of the binding of hemoglobin A0 (Hb), complexed with plasma-derived Hp1-1, recombinant rHp1-1, or rHpr, to immobilized CD163. The concentration of Hp1-1, rHp1-1, and rHpr is shown to the right of each curve.

Fig. 2. Identification of plasma Hpr as a hemoglobin-interacting protein by hemoglobin-affinity precipitation. A, Immunoblot with an anti-Hp antibody (recognizing both Hp and Hpr) of proteins eluted from hemoglobin-coupled Sepharose (Hb), BSA-coupled Sepharose (BSA), or underivatized Sepharose (blank) after incubation with serum from an individual (Hp phenotype 2-1) with a normal Hp level (0.9 g/L) and from a Hp-deficient sickle cell patient. B, Immunoblotting (reducing 12-15% SDS-PAGE) of the hemoglobin-binding serum proteins from an individual with the Hp phenotype 2-1 (same material as shown in Fig. 2A) in order to identify the different $\alpha$-chains of the Hp1, Hp2, and Hpr proteins. Lanes 2, 3, and 4 show silver-stained reducing 12-15%
SDS-polyacrylamide gel of Hp1-1 purified from human plasma, Hp2-2 purified from human plasma, and purified recombinant Hpr, respectively, demonstrating the distinct migration patterns of the Hp1, Hp2 and Hpr α-chains. The identity of the bands from the purified proteins was verified by mass spectrometry (data not shown). C, Silver-stained 8-16% SDS-polyacrylamide gel of proteins from normal human serum bound to hemoglobin-coupled Sepharose or underivatized Sepharose. The identity of the protein bands representing apoL-I and Hpr was determined by mass spectrometry and amino-terminal sequencing (as indicated).
Figure 1, Nielsen et al.
Figure 2. Nielsen et al.
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