Haptoglobin-related protein (Hpr) is a component of a minor subspecies of high density lipoproteins (HDL) that function in innate immunity. Here we show that assembly of Hpr into HDL is mediated by its retained N-terminal signal peptide, an unusual feature for a secreted protein and the major difference between Hpr and the soluble acute phase protein haptoglobin (Hp). The 18-amino acid signal peptide is necessary for binding to HDL and interacts directly with the hydrocarbon region of lipids. Utilizing model liposomes, we show that the rate of assembly and steady-state distribution of Hpr in lipid particles is mediated by the physical property of lipid fluidity. Dye release assays reveal that Hpr interacts more rapidly with fluid liposomes. Conversely, steady-state binding assays indicate that more rigid lipid compositions stabilize Hpr association. Lipid association also plays a role in facilitating hemoglobin binding by Hpr. Our data may offer an explanation for the distinct distribution of Hpr among HDL subspecies. Rather than protein-protein interactions mediating localization, direct interaction with phospholipids and sensitivity to lipid fluidity may be sufficient for localization of Hpr and may represent a mechanism of HDL subspeciation.

A Retained Secretory Signal Peptide Mediates High Density Lipoprotein (HDL) Assembly and Function of Haptoglobin-related Protein*

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Background: Haptoglobin-related protein (Hpr) localizes to a distinct subspecies of high density lipoproteins (HDL).
Results: Hpr binds HDL via a retained signal peptide, is sensitive to lipid fluidity, and requires lipid association for hemoglobin binding.
Conclusion: The signal peptide mediates localization and function of Hpr through direct interaction with HDL lipids.
Significance: Sensitivity to lipid packing may be a mechanism of HDL subspeciation.

Haptoglobin-related protein (Hpr)3 is a primate-specific protein that evolved via a gene duplication event from haptoglobin (Hp), a soluble acute phase protein that mediates the clearance of cell-free hemoglobin (Hb) (1). In contrast to soluble Hp, Hpr localizes to a minor subspecies (<1%) of high density lipoproteins (HDL) that also uniquely contains apolipoprotein L-1 (apoL-1) (2). This HDL particle, termed trypanosome lytic factor-1 (TLF-1), mediates human innate immune protection against many species of African trypanosomes (3). The amino acid sequence of Hpr is greater than 90% identical to Hp (4), the most significant difference being the retention of the N-terminal secretory signal sequence in the mature form of Hpr (5).

The cleavage of N-terminal secretory signal peptides normally occurs in the endoplasmic reticulum. Two other examples of human proteins that retain their N-terminal signal peptide in the mature form have been identified. These proteins, paraoxonase-1 (PON1) and apolipoprotein M (apoM), localize to plasma lipoproteins, mainly HDL (6–8). In both cases, it has been shown that the retained signal peptide is necessary for assembly into HDL (9, 10). The signal peptides of Hpr, apoM, and PON1 are entirely different in amino acid sequence; however, they retain the general characteristics of N-terminal signal peptides, length (18–22 amino acids), a central region of predominantly nonpolar amino acids, a polar N terminus, and a putative C-terminal signal peptide peptidase cleavage site. Despite utilizing a similar mechanism of HDL association, these proteins display different distributions among HDL subspecies (8). Recently, we have shown that the signal peptides from Hpr and apoM adopt different penetration depths in the acyl chain region of lipid bilayers (11). These data may begin to explain the distinct distributions of N-terminally anchored apolipoproteins.

Analysis of the amino acid sequences of PON1 and apoM signal peptides reveal residues in the −1 or −3 position relative to the putative signal peptide peptidase cleavage site that violate von Heijne’s rules for cleavage (12). It has been shown for both proteins that replacing these residues with permissible amino...
Hpr Assembly into HDL

### Table 1

| Peptide | Sequence (N- to C-terminus) |
|---------|-----------------------------|
| Hpr     | SDLGAVISLWGGQR  |
| Hp      | SALGAIVAALLWGRQLF |

Acids results in cleavage (9, 10). It is not as readily apparent why the Hpr signal peptide (Hpr-SP) is not cleaved. Permissible residues are found in both the −1 and the −3 position; however, comparison with the cleaved signal peptide of Hp reveals an arginine insertion at position −5 that may interfere with recognition by the signal peptide peptidase (Table 1). Interestingly, expression of Hpr in an HEK cell system results in cleavage of the signal peptide (4).

Utilizing purified, native Hpr, recombinant Hpr (rHpr) that lacks the signal peptide (4) and a synthetic peptide corresponding to the Hpr signal sequence (Hpr-SP), we define the role of the retained signal peptide in assembly of Hpr into native HDL. Next we utilized model liposomes to examine the role of lipid composition in the binding kinetics and stability of Hpr. Constructing liposomes with defined compositions allowed us to manipulate the physical properties of target lipid particles and address the potential role of physical forces such as van der Waals interactions that may be masked or otherwise difficult to characterize and manipulate in heterogeneous HDL populations. We also show that apol-L-1 may stabilize Hpr association through modulation of lipid packing. Based on these data, and our previous report that Hpr-SP exhibits specificity for fluid mixtures. We also show that apoL-1 may stabilize Hpr association with LDL for 16 h at 37 °C. For LDL scavenging assays, adherent HepG2 cells (ATCC) were grown on glass coverslips in DMEM supplemented with 10% FBS. Coverslips were washed three times with PBS and fixed with 1% paraformaldehyde in PBS for 10 min. Cells were then washed with PBS and incubated in PBS with 100 mM glycine for 5 min. After two additional PBS washes, cells were blocked in 5% BSA and incubated with a 1:100 dilution of a monoclonal antibody against Hpr and 1:1000 dilution of polyclonal anti-SR-B1 (Novus Biologicals) followed by incubation with Alexa Fluor 488- or 633-labeled goat-anti-mouse or goat-anti-rabbit secondary antibodies. For surface localization, trypsinization was performed with 0.25% trypsin for 10 min prior to fixation. Images were acquired with an Axio Observer Z1 equipped with an AxiosCam MRm controlled by AxioVision 4.6 software.

### Experimental Procedures

**Proteins**—Native Hpr and apol-L-1 were purified from human blood as described previously (2, 15). Briefly, blood was collected from healthy fasting donors, and the HDL subfraction was isolated by density gradient ultracentrifugation. Total HDL was solubilized with 10 mM CHAPS, and the delipidated Hpr was purified by one-step immunoaffinity pulldown with monoclonal antibodies against Hpr or apol-L-1 conjugated to agarose beads. Recombinant Hpr cleaved of the signal peptide was obtained from the culture supernatant of transfected HEK 293 cells grown in serum-free media (SFM) (4). Purification of rHpr was accomplished by pulldown directly from media with a monoclonal antibody specific for Hpr (2). Preparations routinely resulted in two bands corresponding to the native Hpr dimer and tetramer, two to four bands for apol-L-1, representing known truncation products, and a single band for the recombinant Hpr dimer when visualized by silver-stained nonreducing SDS-PAGE (data not shown) (2, 4). All proteins were dialyzed into PBS and stored at −80 °C until use.

**Lipids and Peptides**—All lipids and cholesterol (catalog number 700000P) were purchased from Avanti Polar Lipids (Alabaster, AL). These included phosphatidylcholine (PC) from egg (840051), 1,2-dipalmitoyl-sn-glycero-3-PC (DPPC) (850355), 1-palmitoyl-2-oleoyl-sn-glycero-3-PC (POPC) (850457), 1-stearoyl-2-oleoyl-sn-glycero-3-PC (SOPC) (850467), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) (840034), and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (SOPS) (840039). Synthetic Hpr-SP (Table 1) and the tryptophan positional variants were purchased from Bio-Synthesis, Inc. (Lewisville, TX).

**Immunoﬂuorescence Microscopy**—HepG2 cells (ATCC) were grown on glass coverslips in DMEM supplemented with 10% FBS. Coverslips were washed three times with PBS and fixed with 1% paraformaldehyde in PBS for 10 min. Cells were then washed with PBS and incubated in PBS with 100 mM glycine for 5 min. After two additional PBS washes, cells were blocked in 5% BSA and incubated with a 1:100 dilution of a monoclonal antibody against Hpr and 1:1000 dilution of polyclonal anti-SR-B1 (Novus Biologicals) followed by incubation with Alexa Fluor 488- or 633-labeled goat-anti-mouse or goat-anti-rabbit secondary antibodies. For surface localization, trypsinization was performed with 0.25% trypsin for 10 min prior to fixation. Images were acquired with an Axio Observer Z1 equipped with an AxiosCam MRm controlled by AxioVision 4.6 software.

**HDL Scavenging and Binding Assays**—The higher density of Hpr-containing HDL (TLF-1) was taken advantage of to isolate the major fraction of HDL that lacks Hpr (3). The 1.063 g/ml sink fraction of human plasma was adjusted to 1.17 g/ml and ultracentrifuged to resolve the denser Hpr-containing HDL in the float. Analysis of the 1.17 g/ml float by Western blotting with a monoclonal antibody against Hpr or polyclonal anti-Hp that cross-reacts with Hpr reveals no detectable Hpr (see Fig. 1). Low density lipoprotein (LDL) was prepared from total human serum by flotation on 1.063 g/ml NaBr. The Hpr-depleted HDL and LDL were biotinylated with 10 mM EZ-Link sulfosuccinimidyl-NHS biotin (Pierce 21217) in PBS overnight at 4 °C. Excess biotin was removed by dialysis against PBS.

For HDL scavenging assays, adherent HepG2 or HEK 293 cells expressing rHpr cells were grown to ~90% confluency in DMEM + 10% FBS. Cells were washed and incubated in SFM with the addition of 250 μg of biotinylated HDL or biotinylated LDL for 16 h at 37 °C. For *in vitro* HDL binding, purified native or rHpr (585 ng) was incubated with biotinylated HDL (1 μg) in a final volume of 50 μl of PBS at 37 °C overnight.

Biotinylated HDL and LDL were isolated by pulldown with streptavidin-conjugated agarose that had been blocked with 1% bovine serum albumin. The resin was washed in 1× NaCl, and proteins were eluted by solubilizing bound HDL with 10 mM CHAPS in PBS. Supernatant, wash, and pellet fractions were electrophoresed on 10% polyacrylamide gels under nonreducing conditions, transferred to nitrocellulose, and probed with monoclonal anti-Hpr in the case of scavenging assays and polyclonal Hp antibodies that cross-react with Hpr for *in vitro* binding assays. The percentage of bound Hpr (pellet fraction) was normalized to the sum of the pixel intensities across the supernatant, wash, and pellet fractions. Data points are the average of at least triplicate measurements with standard deviations.
Liposome Binding Assays—Liposomes were constructed with DPPC, POPC, or SOPC and 1 mol% biotinylated phosphatidylethanolamine (biotin-PE) (Avanti Polar Lipids, 870277). Large unilamellar vesicles (LUV) were formed by hydration of thin lipid films and extrusion through polycarbonate membranes with 0.1-μm pores. Haptoglobin-related protein binding assays were conducted exactly as described for HDL binding, but with 100 μg of lipid. In the case of apoL-1-LUV binding assays, anionic POPs or SOPs were incorporated into POPC- or SOPC-biotinylated LUV, respectively, at a 1:3 molar ratio, respectively, and incubations with apoL-1 were performed in 50 mM Tris-maleate, pH 5.2, at a 1:100 protein-to-lipid molar ratio. LUV were then diluted into PBS. Signals from no LUV or apoL-1 alone were background-subtracted accordingly.

Liposome Permeabilization Assays—Calcine release assays were performed as described in detail previously (13, 15, 16). Liposomes were extruded above the transition temperature of the component lipids and used immediately to limit the leakage of entrapped dye due to potential changes in phase. The percentage of calcine release was calculated relative to the 100% release value obtained by the addition of 0.01% Triton X-100. Fluorescent traces were obtained with a PerkinElmer Life Sciences LS55 fluorescent spectrophotometer. Temperature was maintained at 37 °C, unless otherwise indicated, with a thermostated biokinetis accessory coupled to an external water bath.

Tryptophan Fluorescence—Fluorescence of the single tryptophan residue in Hpr-SP and three positional tryptophan variant peptides (S1W, WDLGAVISLLLGGRQFLA; S8W, SDLGAVIWTGGLGGRQFLA; A18W, SDLGAVISLLLGGQFLFW) was followed as an indicator of the polarity of the peptide environment. Peptides (500 nM) were incubated in PBS with or without egg PC LUV in PBS for 24 h at 37 °C. Tryptophans were excited at 295 nm, and emission scans were acquired from 320 to 380 nm in the PerkinElmer Life Sciences LS55. Tryptophan quenching was accomplished by the addition of small aliquots of a 5 mM acrylamide solution. Because the upward deviation from linearity is the result of static quenching (17), the Stern-Volmer constants for free peptides were determined from the initial slope of the titration curve (see Fig. 3, dashed line).

Anisotropy Assays—The lipid fluidity of apoL-1-LUV (prepared as described under “Liposome Binding Assays”) was determined by measuring the fluorescence depolarization of diphenyl-1,3,5-hexatriene p-toluenesulfonate (DPMH; Invitrogen D-202) or 1-(4-trimethylammonium-phenyl)-6-diphenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH; Invitrogen, T-204). The anisotropic probes were added to a final concentration of 0.5 mM and allowed to intercalate into the cell membrane for 1 h in the dark. Anisotropic values were acquired via the software function of a PerkinElmer Life Sciences LS55 spectrophotometer. Samples were excited at 358 nm, and emission was read at 430 nm, with 10-nm excitation and emission slit widths. Temperature was maintained at 37 °C by means of the PerkinElmer LS55 biokinetics accessory. Data were corrected for light scattering with an unlabeled sample of LUV, and anisotropy was calculated according to the equation $r = \frac{(I_{VH} - GI_{VH})}{(I_{V} + 2GI_{VH})}$, where $r$ is the anisotropy value, $I_{VH}$ is the emission intensity acquired with the excitation- and emission-polarizing filters set vertically, $G$ is the instrument correction factor, and $I_{V}$ is the emission intensity acquired with the excitation-polarizing filter set vertically and the emission-polarizing filter set horizontally. Data points shown are the average of triplicate measurements with standard deviations.

Hemoglobin Binding Assays—Haptoglobin-related protein binding to Hb was monitored by fluorescence quenching of Hpr tryptophan residues by the heme groups of Hb, as described by Nagel and Gibson (18). Titration of 200 nM Hpr or rHpr in 50 mM sodium phosphate, pH 7.0, was performed with freshly prepared human oxy-Hb. Tryptophans were excited at 280 nm, and fluorescence was monitored at 330 nm. Background fluorescence spectra of Hb alone, or Hb in the presence of HDL or liposomes where appropriate, were subtracted, and inner filter effects were corrected for as described by Hwang and Greer (19).

RESULTS

Hpr Is Presented at the Surface of HepG2 Cells and Can Be Transferred to Exogenous HDL—Assembly of PON1 and apoM can occur through transfer of the mature protein from synthesizing cells to exogenous HDL. For PON1 it has been shown that exchange can take place at the cell surface, and apoM exhibits similar behavior in requiring HDL for secretion (20, 21). Based upon the similarities to mature Hpr, we asked first whether Hpr is localized to the cell surface of HepG2 cells. Probing HepG2 cells with monoclonal anti-Hpr and a polyclonal anti-SR-B1, a known cell surface marker for HepG2 cells, revealed a distinct Hpr signal coincident with the plasma membrane and the signal for SR-B1 (Fig. 1A). The Hpr and SR-B1 signals were sensitive to tryptic digestion, indicating their exposure on the exterior of the cell. Next we asked whether exogenous HDL can serve as an acceptor particle for HepG2-synthesized Hpr. Probing the supernatant SFM from cultured HepG2 cells revealed secreted Hpr in the absence or presence of exogenous HDL or LDL (Fig. 1B). These data are consistent with the finding that apoM is efficiently secreted from HepG2 cells in SFM and is due to endogenous synthesis of apoA-1 and thus production of HDL. Therefore we utilized biotinylated HDL (1.17 float) to isolate exogenous HDL and probed for the presence of Hpr. Western blotting streptavidin-isolated fractions from HepG2 SFM reveals the presence of Hpr in biotinylated HDL but not biotinylated LDL (Fig. 1B). The signal peptide of Hpr is necessary for transfer of Hpr from cell to HDL, but not for secretion of Hpr. This is demonstrated by the absence of Hpr in SFM from rHpr-synthesizing HEK 293 cells, and by the absence of Hpr in the biotinylated HDL fraction.

The Hpr N Terminus Mediates HDL Assembly by Directly Binding Phospholipids—The role of the retained N-terminal signal peptide in Hpr assembly into HDL was determined by performing pulldown assays with native, full-length Hpr and rHpr that lacks the signal peptide. Native Hpr, but not rHpr, bound biotinylated HDL as revealed by Western blotting of supernatant and pellet fractions (Fig. 2A). Native Hpr remains bound after a high ionic strength wash, indicating a stable association with HDL likely via interaction with the lipid hydrocarbon chains.

The lack of evidence for protein-protein interactions that might facilitate Hpr association with HDL (2) and the hydro-
phobicity of the retained signal peptide suggested that Hpr interacts directly with phospholipids. To test this hypothesis we performed binding assays with model liposomes. Native Hpr, but not rHpr, binds egg PC LUV in an ionic strength insensitive fashion (Fig. 2B), consistent with the mode of binding to HDL. The interaction of 100 nM native Hpr with egg PC LUV induces the release of entrapped dye (Fig. 2C), likely through remodeling of the vesicles (15). The apparent lag phase may be indicative of a two-step process as has been demonstrated for the Escherichia coli λ phage receptor signal peptide into lipid bilayers (22). Consistent with the lack of interaction with HDL or LUV, rHpr does not elicit dye release (Fig. 2C).

The Hpr N Terminus Inserts into the Acyl Chain Region Perpendicular to the Acyl Chains—The hydrophobicity of Hpr-SP suggested that association with lipids occurs through interaction with the hydrocarbon chains. We probed the location and orientation of liposome-associated Hpr-SP by determining the peak fluorescence intensities and aqueous accessibility of the single tryptophan in the native peptide sequence and three variant peptides with single tryptophan residues spaced across the length of the peptide, S1W, S8W, and A18W. To maintain a single tryptophan in each peptide and the general hydrophobicity profile of the native peptide, the native tryptophan was replaced by glycine. The addition of increasing amounts of egg PC LUV to solutions of native Hpr-SP resulted in a small blue shift in fluorescence (Fig. 3A). The peak emission intensity of the variant Hpr-SP tryptophans also exhibited small blue shifts (Table 2). Quenching experiments with acrylamide indicate that in the absence of lipid, tryptophans in native Hpr-SP and the positional tryptophan variant peptides are accessible to the aqueous solvent. Stern-Volmer plots indicate that in the presence of egg PC LUV, the native and repositioned tryptophans are shielded, albeit incompletely, from the quenching effects of acrylamide (Fig. 3B, Table 2). The small blue shifts and incomplete shielding of lipid-associated native and variant Hpr-SP indicate shallow penetration and suggest that the peptide adopts an orientation perpendicular to the acyl chains rather than spanning the liposomal bilayer. This interpretation is consistent with our recently published parallax analysis of Hpr-SP orientation in LUV (11).

Binding Kinetics and Steady-state Distribution of Hpr Are Mediated by Lipid Composition—Based upon our previous data that Hpr-SP exhibits specificity for more fluid liposomes (13), we tested whether native Hpr exhibits the same sensitivity to lipid fluidity at physiological temperature. We assayed homogeneous LUV composed of POPC ($T_m = -2 ^\circC$), SOPC ($T_m = 6 ^\circC$), or DPPC ($T_m = 41 ^\circC$) at 37 ^\circC. Calcein release occurred in a biphasic fashion with an initial rapid release rate followed...
by a slower secondary rate. We restricted our analysis to the initial rate to avoid protein/peptide-induced changes in the lipid environment at higher protein-lipid ratios. The rate of Hpr-elicited calcein release is dependent on the composition of the hydrocarbon chains of the lipid bilayer; rates decreased in the order POPC > SOPC > DPPC (Fig. 4A). To determine whether the specificity of Hpr was due to affinity for PC-containing acyl chains with different molecular architecture, we assayed DPPC for susceptibility to Hpr-mediated calcein release at 47 °C, a temperature above the phase transition of DPPC. Native Hpr permeabilizes liquid crystalline DPPC (at 47 °C), but not the more rigid gel phase (at 37 °C) (Fig. 4B).

These data are consistent with our previous findings for Hpr-SP (13) and indicate that it is indeed lipid fluidity that dictates sensitivity to interaction with Hpr rather than a specific binding to certain acyl chains.

Modulation of bilayer properties by mixtures of acyl chains and sterol composition results in significant changes to the intercalation kinetics of Hpr. Titration of DPPC with increasing molar percentages of POPC resulted in increased calcein release from Hpr-SP (Fig. 4C). Although DPPC and POPC exhibit phase separation at 37 °C, the transition temperature of DPPC-enriched domains is decreased with increasing concentrations of POPC (23). Therefore it is likely that the increased rate of dye release is due to both an increase in the surface area of POPC domains and a modulation of the lipid packing of DPPC domains. The addition of membrane-rigidifying cholesterol to POPC liposomes results in a sterol dose-dependent decrease in the rate of Hpr-elicited dye release (Fig. 4D). The effect of increasing concentrations of cholesterol is also apparent when assaying Hpr-SP, supporting the role of Hpr-SP as the mediator of lipid interaction (Fig. 4E).

**ApoL-1 Stabilizes Hpr Association with Lipids—** Apolipoproteins are well known to exchange between lipid environments. Therefore although higher fluidity may allow for faster association kinetics, we asked what effect greater lipid packing, or rigidity, has upon the stability of Hpr-lipid association. We investigated the steady-state distribution of Hpr among the model liposomes employed in the initial rate analysis. Binding assays were performed with POPC, SOPC, and DPPC liposomes by incubating them with Hpr for 24 h at 37 °C. Bound Hpr was visualized by Western blot and quantified by counting pixel density. A greater quantity of Hpr was associated with DPPC than the more fluid POPC or SOPC LUV (Fig. 5).

Ultimately, to define the assembly of human TLF, it is necessary to understand Hpr-lipid interactions in the presence of apoL-1. Based upon the lack of evidence for protein-protein interactions and the ability of Hpr and apoL-1 to bind lipids independent of each other, we asked whether apoL-1 modified lipid packing, and if so, does this influence Hpr binding? First we determined the effect of apoL-1 binding on LUV lipid packing via anisotropy of TMA-DPH and DPH. LUV were constructed with the addition of anionic phospholipids, and binding was performed at acidic pH to facilitate apoL-1 binding (15). Binding of apoL-1 resulted in little or no change in the interfacial or internal rigidity of DPPC: (13) and indicate that it is indeed lipid fluidity that dictates sensitivity to interaction with Hpr rather than a specific binding to certain acyl chains.

Interactions and the ability of Hpr and apoL-1 to bind lipids are well known to exchange between lipid environments. Therefore although higher fluidity may allow for faster association kinetics, we asked what effect greater lipid packing, or rigidity, has upon the stability of Hpr-lipid association. We investigated the steady-state distribution of Hpr among the model liposomes employed in the initial rate analysis. Binding assays were performed with POPC, SOPC, and DPPC liposomes by incubating them with Hpr for 24 h at 37 °C. Bound Hpr was visualized by Western blot and quantified by counting pixel density. A greater quantity of Hpr was associated with DPPC than the more fluid POPC or SOPC LUV (Fig. 5).

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**Lipid Association Facilitates Hpr-Hb Binding—** Hpr was initially thought to lack Hb binding activity (24, 25). Only after analysis of recombinant Hpr lacking the signal peptide was it clear that Hpr bound Hb with high affinity (4). These data,
**FIGURE 4.** 
Hpr exhibits specificity for lipid environments based upon the physical property of lipid fluidity. 

(A) The initial rates of LUV permeabilization by Hpr against POPC (open squares), SOPC (open circles), and DPPC (open triangles) are plotted on a logarithmic scale against increasing protein concentration. Assays were conducted in PBS at 37 °C. 

(B) The refractory DPPC composition was rendered susceptible by thermal fluidization. Representative traces are shown of 200 nM Hpr permeabilization of DPPC LUV at 47 °C, in which the lipids are in a liquid crystalline state, and 37 °C, in which the lipids exhibit more rigid gel phase packing. 

(C) The initial rates of calcein release from POPC:DPPC LUV treated with 1.5 μM Hpr-SP are plotted against the molar concentration of POPC. Error bars indicate mean ± S.D. 

(D) and (E), free cholesterol attenuates interaction of Hpr and Hpr-SP with lipids. Free cholesterol was incorporated into calcein loaded POPC LUV at increasing molar percentages. The ability of 200 nM Hpr (D) or 500 nM Hpr-SP (E) to elicit dye release was assayed in PBS at 37 °C.

**FIGURE 5.** 
Lipid packing stabilizes Hpr association. 

(A) and (B), overnight incubations at 37 °C of Hpr with POPC, SOPC, or DPPC LUV containing 1 mol % biotin-PE were analyzed by Western blotting (A) and quantified by counting pixel density (B). S, supernatant; W, wash; P, pellet; N. L., no lipid. Error bars indicate mean ± S.D.

**FIGURE 6.** 
ApoL-1 may stabilize Hpr-lipid association via increasing lipid packing. 

(A) and (B), the interfacial and bilayer internal rigidity of apoL-1-bound LUV was determined by fluorescence depolarization of TMA-DPH (A) and DPH (B), respectively. Measurements were made at 37 °C. 

(C), overnight incubations at 37 °C of Hpr with apoL-1-associated POPC:POPS or SOPC:SOPS LUV containing 1 mol % biotin-PE were analyzed by Western blotting and quantified by counting pixel density. Error bars indicate mean ± S.D.
along with our inability to pull down delipidated, native Hpr with an Hb affinity resin (data not shown), led us to test the role of lipids in modulating Hpr-Hb binding. The lack of fluorescence quenching of Hpr by Hb indicates that native, delipidated Hpr does not bind Hb (Fig. 7A). Hemoglobin binding by Hpr can be stimulated by the addition of non-Hpr-containing HDL (Fig. 7B). Stimulation of Hb binding activity is mimicked by the addition of LUV (Fig. 7B). Consistent with previous SPR analysis (4), rHpr, purified in a similar fashion to native, delipidated Hpr, exhibits Hb binding capacity (Fig. 7C). Exogenous HDL does not further stimulate Hb binding by rHpr. These data indicate that HDL facilitate Hpr-Hb binding via lipid interaction with the signal peptide.

**DISCUSSION**

Assembly of Hpr into HDL is mediated by the retained N-terminal secretory signal peptide. Retention of the Hpr signal peptide is not fully explained by violation of von Heijne’s rules for signal peptide cleavage, in which small, neutral amino acids are necessary at positions −1 and −3 relative to the putative cleavage site (12). Comparison with the similar, cleaved Hp signal peptide suggests that an arginine at position −5 may inhibit cleavage (Table 1). There is precedence for a large, charged amino acid at position −5 inhibiting signal peptide cleavage. Bovine CD18 retains the signal peptide due to a glutamine residue at position −5. Replacement of glutamine with glycine at position −5 in the bovine protein restores signal peptide cleavage (26). Bovine CD18 remains associated with the cell due to a transmembrane region. Despite possessing the bona fide Hpr signal sequence, rHpr is cleaved of its signal peptide when expressed in HEK cells in vitro (4). It is therefore a possibility that signal peptide cleavage is dependent on cell type and that a soluble isoform of the protein exists in plasma.

The Hpr-SP is necessary for association of Hpr with HDL. Binding of Hpr to LUV suggests that the lipid component of HDL is sufficient and that Hpr does not require ancillary HDL proteins for assembly into TLF-1. Despite previous attempts to detect protein-protein interactions that might direct TLF-1 assembly, no evidence exists for direct interaction of Hpr with apol-L-1. Additionally, solubilization with mild nonionic detergents abolishes co-immunoprecipitation of apoA-1, apoL-1, and Hpr, supporting a central role for lipids in the structure and stability of TLF-1 (2). These data raise the question of how the majority of Hpr localizes to a minor subclass of HDL particles that also contain apoL-1. One plausible model may be that association of apol-L-1 causes changes to the HDL physical properties that in turn influence the binding of Hpr. Indeed, apol-L-1 influences Hpr binding as discussed below. Our data also allow for a model in which Hpr and apol-L-1 interact intracellularly or at the cell surface and concomitantly assemble into circulating HDL where protein-lipid interactions subsequently provide the dominant stabilizing forces. It is interesting to note that Hpr, apoa-1, and apol-L-1 also colocalize in a large, lipid-poor serum complex termed TLF-2 that also contains IgM (25). The lack of lipids in TLF-2 offers a scenario in which protein-protein interactions may provide the dominant stabilizing forces.

Multiple model systems, including triolein emulsions (27), phospholipid monolayers (28), and lipid vesicles (29), have been utilized to gain information about apolipoprotein-lipid interactions. Multiple factors, including acyl chain composition, cholesterol content, protein composition, and surface curvature, may contribute to the degree of lateral van der Waals interactions in HDL. The complex protein and lipid composition within an HDL particle in addition to the heterogeneity of HDL populations limits experimental approaches to study the physical forces involved in

![Figure 7. Lipid association facilitates Hpr-Hb binding.](image-url)
Hpr Assembly into HDL

native HDL dynamics. The simplified LUV system allowed us to identify the influence that lipid fluidity has upon the kinetics and stability of Hpr binding. We suggest that simplified, homogeneous LUV are valuable tools for studying the apolipoprotein-lipid interactions that are relevant to HDL assembly and subspeciation. Additionally, LUV have been successfully employed as HDL mimetics to scavenge N-terminally anchored apolipoproteins from the surface of synthesizing cells (10), further supporting the validity of LUV as models for HDL.

Dye release assays indicate that the kinetics of Hpr intercalation into the hydrocarbon regions of LUV are a function of lipid packing. Intercalation of Hpr is significantly slower into liposomes composed of phosphatidylcholines with longer or saturated acyl chains. The slower kinetics of Hpr intercalation into SOPC than POPC at 37 °C indicate that minor differences in acyl chain length can have a significant effect upon the rate of Hpr intercalation at physiological temperature. Due to the cooperativity of lipid packing, titrating unsaturated POPC into the more rigid DPPC liposomes increases the rate of Hpr intercalation. Although these lipid mixtures do not approach the complexity of acyl chain compositions in native lipoproteins, the data do indicate that acyl chain heterogeneity may play a role in dictating assembly of Hpr into HDL having specific physical properties. Unesterified cholesterol concentration also modulates the rate of Hpr intercalation into lipid particles. The decreased rate of dye release observed with increasing molar percentages of cholesterol incorporated into POPC LUV can be attributed to rigidification of lipid packing. These data identify another variable of HDL, free cholesterol content, as a physiologically relevant potential mediator of Hpr assembly into specific HDL particles.

Although the kinetics of LUV interaction are faster for more fluid lipid compositions, the steady-state binding of Hpr to LUV is greater for more tightly packed lipids. This finding is recapitulated in a more physiologically relevant system when apoL-1 is incorporated into LUV. Our data indicate that apoL-1 increases the packing of the hydrocarbon region in LUV, and we see a concomitant increase in the steady-state levels of bound Hpr. It is therefore plausible that apoL-1 stabilizes Hpr within TLF by decreasing the dissociation rate through increased van der Waals forces. It may also be the case that Hpr plays a role in the architecture and stability of associated HDL. Previously, we have shown that Hpr remodels target 100-nm-diameter LUV, resulting in both larger (>200-nm) and smaller (10–50-nm) lipid particles (15). Therefore dye release from LUV may be the result of fission/fusion of the vesicles.

N-terminal anchoring of apolipoproteins, exemplified by apoM, PON1, and now Hpr, is a relatively unexplored mechanism of HDL association. Shotgun proteomic analysis of human HDL identified greater than 100 associated proteins. These data, and the large percentage of HDL surface area occupied by apoA-1 (30), raise the question of how so many proteins might associate with a particle that offers only limited lipid surface area. Although protein-protein interactions may facilitate association of some of these proteins, we have shown that Hpr association does not require ancillary proteins to bind lipidic particles. Anchoring a protein into HDL lipids via a small peptide such as Hpr-SP, rather than extended amphipathic helices, offers an association mechanism that may not require large surface areas.

The penetration depth of Hpr-SP is apparently limited to the outer leaflet of LUV, suggesting that in the case of native HDL, Hpr may have little or no direct interaction with the hydrophobic core. The shallow penetration and perpendicular orientation, relative to the acyl chains, of Hpr-SP is reminiscent of the amphipathic helical mode of interaction employed by a variety of apolipoproteins. Indeed, there is segregation of polar and nonpolar residues when Hpr-SP is visualized by helical wheel projection, and CD spectroscopy indicates a predominantly α-helical structure in lipid (11). The similarities with an amphipathic mode of lipid association may reflect a common mechanism of apolipoprotein exchange between lipid environments.

Secretion of apoM and PON1 into the circulation requires the presence of an acceptor lipid particle (20, 21). For apoM it has been shown that endogenous synthesis of HDL facilitates secretion in HepG2 cells (21). Similarly, we have localized Hpr to the surface of HepG2 cells and find that Hpr is secreted into SFM in the absence of exogenous lipoproteins. To discriminate between cellular assembly of Hpr into HDL and subsequent secretion and transfer of Hpr from cells to exogenous HDL, we incubated HepG2 cells with biotinylated lipoproteins, isolated these with streptavidin-agarose pulldown, and probed for the presence of Hpr. We observed Hpr in the isolated HDL but not LDL fraction, indicating 1) that Hpr can be transferred from the surface of HepG2 cells to circulating HDL and 2) that there is a specificity for HDL transfer over other lipoproteins. This specificity may be due to cellular factors, lipoprotein properties, or combinations thereof. James et al. (31) have demonstrated a role for the HDL scavenger receptor class B, type I (SR-BI) in facilitating PON1 assembly into HDL. Decreased expression of SR-BI in hepatocytes resulted in increased retention of PON1 at the cell surface. It may be the case that SR-BI serves as a molecular dock for HDL and/or facilitates the local assembly of necessary cell surface lipids or other components. Specific HDL structural features are also apparently involved. A study published by Deakin et al. (20) reported that smaller reconstituted HDL (9.6 nm) were less efficient than larger reconstituted HDL (17 nm) at inducing release of PON1 from cell surfaces. Additionally, the incorporation of unesterified cholesterol diminished the capacity to release PON1 (20). The size of HDL and cholesterol content can be determinants of lipid fluidity, with smaller, discoidal HDL or higher cholesterol content conferred less fluidity. The native HDL subspecies containing Hpr, TLF-1, is relatively large, 15–20 nm in diameter, and it contains ~10% cholesterol by weight (3). It is not known whether this facilitates, or is a consequence of, Hpr association.

It has long been known that lipid fluidity plays a role in the assembly of apoA-1-lipid complexes (32). Additionally, the acyl chain composition of reconstituted HDL influences the efficiency of cellular cholesterol uptake, with more fluid compositions conferring faster efflux velocity (33). Our data suggest that lipid fluidity is also an important factor in the assembly of Hpr, and most likely other N-terminally anchored apolipoproteins, into HDL. The dynamics of HDL have been well studied with respect to the maturation of nascent lipid-poor apoA-1, discoidal HDL and triglyceride, and esterified cholesterol-containing.
spherical particles (34). These different structural forms govern physiological interactions of HDL. The lipid-poor form of apoA-1 is preferentially bound by the ATP-binding cassette transporter A1 (35), involved in lipidation of apoA-1, whereas the discoidal form is most efficient at activating the lecithin:cholesterol acyltransferase enzyme (36), and the spherical form is the major substrate for SR-B1 (37). However, particularly in light of the recent proteomic data (6, 8), it is not understood how these different morphologies might contribute to HDL subpecification. We suggest that at least one factor contributing to subpecification is lipid fluidity. Whether this occurs through protein-mediated restriction of lipid motion, or lipid and sterol composition of the particle itself, our model-based data suggest that lipid particles can be susceptible or refractory to association with specific apolipoproteins. It is also important to point out that our data do not address the specificity of Hpr for HDL rather than other circulating lipoproteins such as LDL, intermediate density lipoprotein (IDL), or VLDL. Again, this specificity may be governed by a variety of other physiological factors such as different receptor interactions at the cell surface or even different metabolic fates of the lipoproteins. Incorporating the role of SR-B1 as a cell surface docking molecule (31) and our data concerning the steady-state distribution of Hpr, we can suggest a scenario for the assembly of N-terminally anchored apolipoproteins into HDL: 1) binding of HDL to SR-BL, 2) transfer of the N-terminal anchor from the cellular lipid bilayer to a sufficiently fluid HDL, and 3) redistribution of the N-terminally anchored apolipoprotein into a lipid environment that is suitably fluid to allow insertion, but also sufficiently rigid to retain the protein. The latter step may be a result of the constant metabolic remodeling of HDL by a variety of factors (38). Regarding this last point, it has been shown that PON1 exchange between HDL and the external surface of cells is not unidirectional. Redistribution of PON1 to distal endothelial cells has been demonstrated (39). Perhaps most interesting to note is that despite similar mechanisms of association with HDL, PON1, apoM, and Hpr exhibit distinct distributions among HDL subspecies (8). A systematic determination of the surface fluidity of HDL subspecies may be a useful resource for understanding subpecification and thus alternative functions of HDL.

Lipids modulate Hb binding by Hpr. Immunoaffinity-purified native Hpr that has been delipidated does not bind Hb. Providing exogenous lipid in the form of HDL or LUV may stimulate Hb binding by preventing the signal peptide from interfering directly with the Hb-binding site or allosterically changing Hpr structure. Hb binding is a necessary step for innate immune-mediated killing of Trypanosoma brucei (14). These data may explain the failure of early attempts to demonstrate Hb binding by Hpr or TLF-1 (24, 25). Delipidated PON1 also exhibits attenuated enzymatic activity, a factor that may contribute to the pathology of type 2 diabetes (40). Removal of signal peptides from soluble proteins may be a general housekeeping task that facilitates protein structure/function.

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