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Hepcidin, the hormone of iron metabolism, is bound specifically to α-2-macroglobulin in blood

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Hepcidin is a major regulator of iron metabolism. Hepcidin-based therapeutics/diagnostics could play roles in hematology in the future, and thus, hepcidin transport is crucial to understand. In this study, we identify α_2-macroglobulin (α_2-M) as the specific hepcidin-binding molecule in blood. Interaction of ^{125}I-hepcidin with α_2-M was identified using fractionation of plasma proteins followed by native gradient polyacrylamide gel electrophoresis and mass spectrometry. Hepcidin binding to nonactivated α_2-M displays high affinity (K_d 177 ± 27 nM), whereas hepcidin binding to albumin was nonspecific and displayed nonsaturable kinetics. Surprisingly, the interaction of hepcidin with activated α_2-M exhibited a classical sigmoidal binding curve demonstrating cooperative binding of 4 high-affinity (K_d 0.3 μM) hepcidin-binding sites. This property probably enables efficient sequestration of hepcidin and its subsequent release or inactivation that may be important for its effector functions. Because α_2-M rapidly targets ligands to cells via receptor-mediated endocytosis, the binding of hepcidin to α_2-M may influence its functions. In fact, the α_2-M–hepcidin complex decreased ferroportin expression in J774 cells more effectively than hepcidin alone. The demonstration that α_2-M is the hepcidin transporter could lead to better understanding of hepcidin physiology, methods for its sensitive measurement and the development of novel drugs for the treatment of iron-related diseases. (Blood. 2009;113:6225-6236)

Introduction

The hormone hepcidin plays a role in orchestrating iron metabolism.[1-12] This peptide, originally discovered in urine as a bactericidal molecule,[13] was later shown to be a regulator of iron metabolism.[3,5] In fact, it plays an important role in conditions of altered iron demand.[3,5]

The function of hepcidin is regulation of transmembrane iron transport.[4,5] Hepcidin binds to its cell surface receptor, ferroportin (solute carrier family 40 [iron-regulated transporter], member 1), leading to internalization and degradation of the protein complex by the lysosome.[1] Because ferroportin enables iron efflux from enterocytes, hepatocytes, and macrophages, its internalization after hepcidin binding leads to decreased iron release.[1] Hence, the hepcidin-mediated decrease in ferroportin iron export from enterocytes into blood leads to depressed intestinal iron absorption.[3,4] At the same time, iron export from hepatocytes and macrophages is blocked, which further decreases serum iron.[3-5]

Hepcidin also plays a role during inflammation, infection, and cancer.[3-5] Under these conditions, iron is shifted from the circulation into stores, making it less available.[3] In anemia and hypoxia, hepcidin regulates iron availability for erythropoiesis. In the future, hepcidin may find a place in treating disease states.[3-5] Furthermore, hepcidin is predicted to become an indicator of body iron stores.

We investigated the presence of plasma hepcidin-binding molecules because the identification of such entities will provide new insights into hepcidin function. Indeed, it is known that many hormones are transported in the blood by carrier molecules,[14-16] but a specific hepcidin-binding protein has not been identified. Hence, knowledge of hepcidin transport is essential for understanding its distribution and may be important for its measurement in plasma.

In this study, we incubated blood plasma with ^{125}I-labeled human hepcidin and then separated plasma proteins using native electrophoresis. For the identification of these molecules, the complexes were purified using a native 2-dimensional separation technique with identification by mass spectrometry.[17] We identified α_2-macroglobulin (α_2-M) as the specific hepcidin-binding molecule in plasma.

Methods

Chemicals, plasma, and serum

Chemicals were from Sigma-Aldrich (St Louis, MO). Blood was obtained from healthy volunteers (2 females, 3 males) after the study was approved by the Ethics Committee (Institute of Hematology and Blood Transfusion, Prague) and informed consent was obtained from the participants in

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Table 1. Serum Fe, transferrin saturation, and serum ferritin levels in blood from healthy volunteers in this study

Healthy volunteers	Serum Fe,* μmol/L	Tf saturation,† %	Serum ferritin,‡ μg/L
Female, A	10.86	35	75
Female, B	22.32	41	55
Male, A	9.14	50	98
Male, B	25.41	26	79
Male, C	16.45	35	25
Mean ± SD	16.84 ± 5.75	37.4 ± 7.22	66.4 ± 22.64

Reference values from Shinton[18] and Bick.[19]
Tf indicates transferrin.
*Normal values: female (7.16-26.85 μmol/L), male (8.95-28.64 μmol/L).
†Normal values: female and male (20%-55%).
‡Normal values: female (12-150 μg/L), male (15-150 μg/L).

accordance with the Declaration of Helsinki. To obtain serum, blood was collected into vacuutainer tubes, allowed to stand 15 minutes and centrifuged (2000*g* for 25 minutes at 20°C). To obtain plasma, blood was collected into vacuutainer tubes and centrifuged (1500*g* for 5 minutes at 20°C). Plasma or serum were pooled and used for experiments. Analysis of these blood samples by the Institute of Hematology and Blood Transfusion demonstrated they were within the normal range for serum iron, serum ferritin, and transferrin saturation (Table 1[18,19]).

Reagents

Unmodified (DTHFPICIFCCGCCHRSKCGMCCKT) and M21Y-modified (DTHFPICIFCCGCCHRSKCGYCCKT) hepcidin peptides were custom-synthesized and purity was confirmed by Clonestar Peptide Services (Brno, Czech Republic). Goat anti–human α_2-M antibody (sc-8514) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Hepcidin renaturation

We used an established method for hepcidin renaturation based on dissolving hepcidin in denaturant (6 M guanidine HCl), subsequent reduction with dithiothreitol, followed by purification using reversed-phase chromatography and slow oxidation.[1]

Hepcidin concentration

We compared 3 methods for determining hepcidin concentration: (1) absorbance difference at 215 to 225 nm[20]; (2) quantitative determination of sulfhydryl groups[21]; and (3) measurement of absorbance at 205 nm.[22] All methods yielded the same concentration, and, subsequently, method 1 was used.

Radiolabeling of hepcidin

Hepcidin was labeled with ^{125}I via the protocol of Nemeth et al[1] using IodoBeads (Pierce Biotechnology, Rockford, IL). This protocol maintains the conformation of hepcidin.[1] Iodinated hepcidin was then purified using a Waters μBondapack C18 WAT027324 column (Waters, Milford, MA) prewet with methanol and equilibrated with 0.1% trifluoroacetic acid (TFA).

Determination of biologic activity of renatured hepcidin

The biologic activity of hepcidin was determined according to Rivera.[23] Twelve to 14 days before the experiment, 5-week-old female C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) were switched to a low-iron diet (Harlan-Teklad, Madison, WI). These were injected intraperitoneally with 50 μg hepcidin, controls were injected with phosphate-buffered saline (PBS). Six hours later, the mice were anesthetized with avertin and blood was collected. The blood was centrifuged at 700*g* for 15 minutes at 4°C and the sera collected. Serum iron and total iron-binding capacity were then analyzed (Jewish General Hospital, Montreal, Canada). Animal studies were performed with approval of the Animal Care Committee of the Lady Davis Institute for Medical Research/McGill University, Montreal, QC.

Fractionation of blood plasma proteins by FPLC

Fractionation was performed at 25°C. Blood plasma (500 μL) was diluted with 4.5 mL buffer A (50 mM Tris-HCl, pH 8.0) and loaded onto a MONO Q-5/50 GL column (GE Healthcare, Little Chalfont, United Kingdom) connected to a BioLogic HR (Bio-Rad Laboratories, Hercules, CA) fast-pressure liquid chromatography (FPLC). The fractions were eluted using a linear gradient (0-1 M) of NaCl in buffer A.

Native gel electrophoresis and gel processing

Samples were separated on a linear gradient (3%-12%) native polyacrylamide gel containing Triton X-100 in Tris-glycine buffer.[17] The gel was exposed and scanned on a phosphorimager (Fuji, Cypress, CA). The radiogram was analyzed using Aida software (Raytest, Straubenhart, Germany).

Analysis of the hepcidin–α_2-M interaction using FPLC

Binding of hepcidin to plasma proteins or purified α_2-M was studied via size-exclusion chromatography using FPLC (BioLogic DuoFlow System; BioRad) fitted with a Superdex 200 10/300 GL (GE Healthcare) column. Samples of plasma or purified α_2-M (Sigma-Aldrich) were incubated with ^{125}I-hepcidin for 1 hour at 37°C, loaded into the column and eluted with 0.14 M NaCl/0.01 M Hepes (pH 7.4) at 25°C. Radioactivity in the fractions was measured with a 1480 Wallac Wizard 3′ gamma counter (Turku, Finland).

Enzymatic in-gel digestion and μLC-mass spectrometric analysis

In-gel digestion and mass spectrometric analysis was performed as described.[17] Proteins were reduced with 30 mM Tris-(2-carboxyethyl)phosphine hydrochloride at 65°C for 30 minutes and alkylated by 30 mM iodacetamide for 60 minutes in the dark. After overnight digestion at 37°C in buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 10% MeCN, and sequencing grade trypsin (20 ng/μL; Promega, Madison, WI), the peptides were purified on a macrotrap column packed with polymeric reversed-phase material (Michrom BioResources, Auburn, CA). The column was connected to an LCQ^{DECA} ion trap mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray ion source. Full scan spectra were recorded over 350 to 2000 Da followed by MS/MS scans of the 3 most intense ions in the preceding full scan. Peak lists were analyzed using SEQUEST (Thermo Fisher Scientific, Waltham, MA).

Activation of α_2-M by methylamine

Protease-binding to α_2-M triggers a conformational change converting it to an activated form able to bind hormones.[24] Proteases cannot be used to activate α_2-M due to their damaging effects, which would prevent proper binding analysis. Considering this, previous workers demonstrated this conformational change can be mimicked by treatment with methylamine.[24-26] Furthermore, physicochemical and functional data suggest that methylamine activated α_2-M (α_2-M-MA) closely resembles the structure and function of protease-activated α_2-M.[25,26]

In vitro conversion of α_2-M by methylamine into the activated protein (α_2-M-MA) was achieved using an established technique where samples of α_2-M (1.4 μM; Sigma-Aldrich) were treated with 200 mM methylamine/0.05 M Tris/HCl (pH 8.1).[27] Unreacted methylamine was removed from α_2-M-MA via a Sephadex G-25 centrifugal column.[28,29]

Determination of α_2-M and α_2-M-MA-hepcidin complex stoichiometry by ultracentrifugation

α_2-M (Mw 720 000 Da) can be sedimented by ultracentrifugation.[30] We exploited this method to determine α_2-M-hepcidin and α_2-M-MA-hepcidin complex stoichiometry.

The mixture of α_2-M or α_2-M-MA with hepcidin (molar ratio, 1:5; at this molar ratio hepcidin is above the α_2-M and α_2-M-MA saturating concentration) was incubated at 37°C for 2 hours and then subjected to ultracentrifugation at 180 000*g* for 2 hours at 37°C. Control tubes contained hepcidin only. The amount of hepcidin bound to α_2-M or α_2-M-MA was

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calculated by subtracting the hepcidin concentration in the supernatant before and after ultracentrifugation. The number of hepcidin binding sites was calculated as the molar ratio of the bound hepcidin to total α_2-M. In preliminary experiments using the ultracentrifugation of α_2-M and α_2-M-MA without any hepcidin added, we proved by measurement of total protein (Quick Start Protein Assay Kit; Bio-Rad) that no α_2-M remained in the supernatant after ultracentrifugation, as it was completely pelleted. We also verified that the hepcidin concentration in the control tubes was the same before and after centrifugation.

Determination of the K_d for hepcidin-α_2-M and hepcidin-α_2-M-MA

To determine the equilibrium dissociation constant (K_d) for hepcidin-binding to α_2-M, samples containing a constant amount of α_2-M or α_2-M-MA and increasing concentrations of ^{125}I-hepcidin were incubated for 2 hours at 37°C and separated using native electrophoresis. The radiogram was quantified using Aida software (Raytest).

Under our conditions, the determination of K_d from the binding curve required correction for hepcidin depletion. Assuming that nonactivated α_2-M contains 2 binding sites for hepcidin (as indicated from ultracentrifugation experiments), we calculated the free hepcidin concentration for each well by subtraction of the bound hepcidin obtained from the y-axis values. The binding hyperbola were processed using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA).

The binding data for α_2-M-MA were fitted using a multisite Hill equation.[31] The free hepcidin concentration was calculated using the numerical solution of an implicit binding function (see equations 1-3). The data analysis was based on the *concerted* binding reaction:

$$M + n\,H \leftrightarrow MH_n \quad (1)$$

with the dissociation equilibrium constant:

$$K_d^n = \frac{[M][H]^n}{[MH_n]} \quad (2)$$

where M denotes α_2-M; H, hepcidin; n, the cooperativity coefficient; and the square brackets denote concentration. We derived the modified Hill equation, that is expressed in terms of the total concentrations (hence subscript 0) of hepcidin and macroglobulin:

$$Y = \frac{([H]_0 - nY[M]_0)^n}{K_d^n + ([H]_0 - nY[M]_0)^n} + B \quad (3)$$

where Y is the ratio of the concentration of the hepcidin-α_2-M complex to that of total α_2-M. The fitting of equation 3 to the data involved iteration of the values of n and K_d combined with the numerical solution of equation 3, using *NSolve* in *Mathematica*.[32] Refinement of the choices of values for n and K_d were made by inspection of fit of the function to the data. The use of nonlinear regression was not possible because of the implicit (in Y) nature of equation 3.

Cell culture and Western analysis

J774 cells (ATCC) were grown using standard procedures[33] and used when at approximately 90% confluence to maximize ferroportin detection. Western analysis was performed by established methods[34,35] using the Invitrogen NuPAGE Novex System for optimal detection (Carlsbad, CA). Briefly, protein samples (100 μg) were separated on NuPAGE Bis-Tris 4% to 12%, 1.5 mm gels (Invitrogen) and then transferred to Invitrolon PVDF membranes. The primary antibodies used were anti-ferroportin (1/2500; Dr D. Haile, Audie Murphy Hospital, San Antonio, Texas) and anti–β-actin (1/10 000; clone AC-1; Sigma-Aldrich). Secondary antibodies used were anti–rabbit (1:2000; Sigma-Aldrich) and anti–mouse (1:10 000; Sigma-Aldrich) conjugated with horseradish peroxidase. Incubation with the primary antibody was for 2 hours at room temperature (RT), and with the secondary antibody for 1 hour at RT both in 10% skim milk (Tris-buffered saline/0.1% Tween 20; pH 7.4). The protein bands were visualized using ECL (GE Healthcare). Bands on x-ray film were quantified by scanning densitometry and analyzed using Quantity One (Bio-Rad).

Statistical analysis

Results were expressed as mean plus or minus SD. Data were compared using the Student *t* test. Results were considered significant for *P* values less than .05.

Results

^{125}I-labeling of native and M21Y hepcidin

We hypothesized that similar to other hormones, hepcidin circulates bound to specific binding protein(s).[16,36] We examined the presence and identity of binding proteins using ^{125}I-hepcidin and native nondissociative separation techniques.

Synthetic hepcidin must be renatured to be a soluble and effective signaling molecule. This was performed using established methods and the biologic activity tested using mice.[1] Active hepcidin can be labeled by ^{125}I and used as a tracer. Because tyrosine, which is considered to be optimal for efficient radio-iodination, is not present in natural human hepcidin, modified hepcidin with a Met/Tyr[23] substitution (M21Y modified) was also assessed, as in previous studies.[1,6] This modified hepcidin was compared with unmodified (physiologically relevant) hepcidin.

In preliminary experiments, we compared the ^{125}I-labeling efficiency of both unmodified hepcidin (DTHFPICIFCCGCCHRSKCGMCCKT), containing 2 histidines and 2 phenylalanines, which can be iodinated,[37] and the M21Y-modified human hepcidin. We performed 2 parallel labelings of the same amount of either peptide using equal amounts of ^{125}I. Interestingly, both forms of hepcidin were labeled with similar specific activity (unmodified hepcidin, 1.6 μCi/μg; modified hepcidin, 2.1 μCi/μg). Hence, under these labeling conditions, tyrosine modification of hepcidin only slightly increased bound ^{125}I.

To avoid the possible problem that M21Y-modified hepcidin may behave differently from unmodified hepcidin, we used only unmodified (physiologically relevant), ^{125}I-labeled, HPLC-purified (Figure 1A) human hepcidin in all further experiments described below. Importantly, this peptide was shown to display physiologic activity by significantly ($P < .01$) decreasing the saturation of transferrin with iron after it was injected into mice (Figure 1B), demonstrating its utility for further studies. There was no difference in the ability of labeled or nonlabeled hepcidin to decrease transferrin iron saturation, demonstrating the functional integrity of the peptide.

Identification of 2 hepcidin-binding proteins

After incubation of human plasma or serum with ^{125}I-hepcidin and subsequent separation using nondenaturing native electrophoresis, 2 major bands (labeled as "?") were detected corresponding to complexes of ^{125}I-hepcidin with unknown molecules (Figure 2A). The migration of the 2 bands on Figure 2A are markedly different, at least in part, probably due to their differences in M_r. Free hepcidin is not visible, because as a cationic peptide, it migrates out of the sample well toward the cathode. For purification of these protein complexes and their identification by mass spectrometry, we required a nondenaturing separation technique that preserves ^{125}I-hepcidin-binding protein interactions and enables high resolution separation of the many plasma

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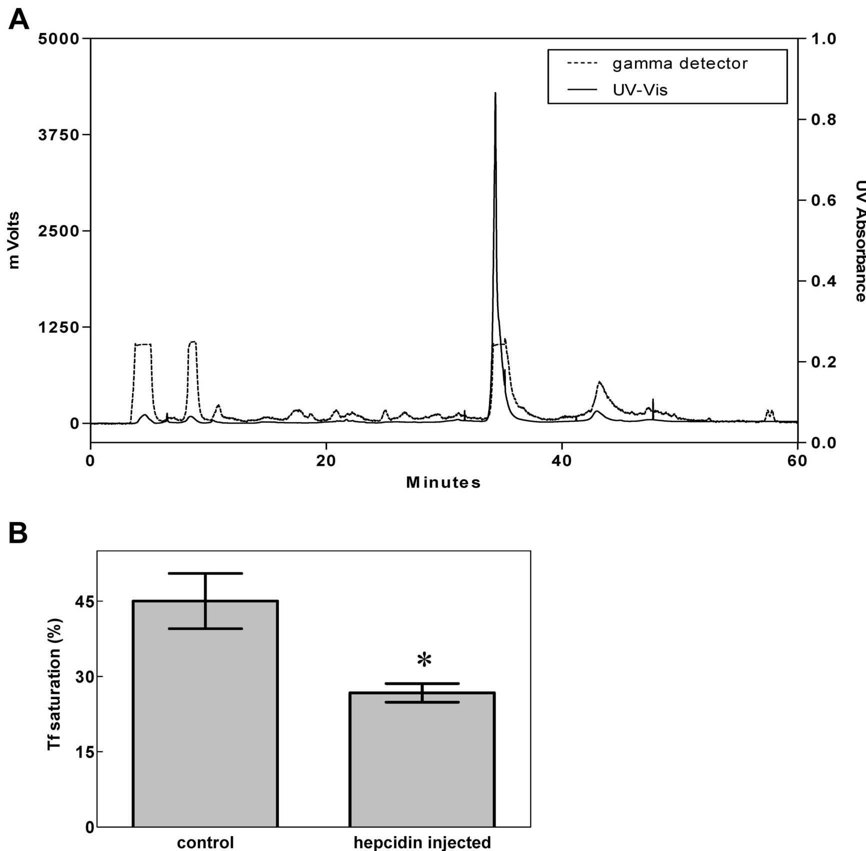

Figure 1. Preparation of bioactive hepcidin. (A) The HPLC profile of purified ^{125}I-hepcidin. Synthetic human hepcidin was renatured, labeled using ^{125}I, as described in "Methods," and subjected to HPLC purification (broken line: ^{125}I radioactivity; solid line: 210 nm UV absorbance). More than 98% of the peptide was present in the main ^{125}I peak. (B) Hepcidin significantly (*$P < .01$) decreases the saturation of transferrin (Tf) with iron when mice were injected with the renatured peptide. Results in panel A are typical of 3 or 4 experiments, while those in panel B are mean ± SD (3-4 experiments).

proteins. To meet these requirements, we used an established nondenaturing, 2-dimensional separation technique to identify the hepcidin-binding plasma protein(s).[17] This method combines FPLC with native electrophoresis and is compatible with mass spectrometry.[17]

Fractions of plasma from the first dimension of the preparative separation using FPLC (Mono Q column; Figure 2B) were collected, and aliquots from each were trace labeled with ^{125}I-labeled hepcidin with which it was incubated for 1 hour at 37°C. The labeled fractions were then separated by native 3% to 12% gradient polyacrylamide gel electrophoresis (PAGE) as a second dimension of separation (Figure 2C). Fraction 13 contained only the lower, rapidly migrating band in Figure 2A, while fractions 15 to 17 contained both the lower and top bands (Figure 2C). Thus, the latter fractions were pooled and used for further analysis, described in the next section below, to identify the ^{125}I-hepcidin-binding molecules. It was of interest to note that the upper band in fraction 15 consisted of a doublet (Figure 2C). This could correspond to the activated and nonactivated forms of α_2-M that are known to migrate slightly differently[24,38] (see also Figure 7).

Examination of the hepcidin-binding proteins in blood

To identify the uppermost hepcidin-binding protein shown on Figure 2A, fractions 15 through 17 in Figure 2C were concentrated on centrifugal ultrafilters (Millipore Ultrafree NMW 3000) and resolved by native 3% to 12% gradient PAGE. When an aliquot of the pooled fractions was separated by native gradient PAGE and stained for protein with Coomassie blue (Figure 3A lane 1), the top band was in a similar migration position as the ^{125}I-hepcidin-binding molecule detected by phosphorimaging after a 1 hour incubation of ^{125}I-hepcidin with either human plasma (Figure 3A lane 2) or pooled fractions 15 through 17 (Figure 3A lane 3). The band resulting from the addition of ^{125}I-hepcidin to plasma (Figure 3A lane 2) or pooled fractions 15 through 17 (Figure 3A lane 3) comigrated with a band resulting from the incubation of ^{125}I-hepcidin with purified α_2-M for 1 hour at 37°C (Figure 3A lane 4). It is important to note that the uppermost band was well resolved from adjacent proteins, allowing subsequent MS analysis (described in the next paragraph).

Guided by the phosphorimager scan, the top band in Figure 3A (lane 3) was cut from the gel. This band corresponded to a complex of ^{125}I-hepcidin with an unknown protein from fractions 15 through 17. This segment of gel was rehydrated, digested with trypsin, and subjected to MS analysis. The unknown protein was unambiguously identified by μLC-MS/MS analysis as α_2-M (P01023; Table 2). A SEQUEST search against the human SwissProt database assigned 55 peptides to the protein covering 52% of the α_2-M sequence (Figure 3B,C; Table 2). It is notable from Table 2 that other proteins were also identified, although the abundance was far lower (number of peptides: 2-6). These comigrating molecules were also tested for ^{125}I-hepcidin-binding, but no significant affinity was identified (data not shown). Hence, considering the low abundance of these other proteins in comparison to α_2-M and their lack of hepcidin-binding ability, they were considered contaminants.

To further confirm that α_2-M was a specific hepcidin-binding protein, we performed α_2-M immunoprecipitation experiments on human plasma followed by stripping ligands from α_2-M with strongly acidic buffer and ultrafiltration of released peptides on a Millipore Ultrafree filter, M_r cutoff 5 kDa. We proved by mass spectrometry the presence of hepcidin in the ultrafiltrate. Other peptides, known to be bound by α_2-M, were also identified, such as osteoprotegerin.[14] The detectability of hepcidin in MG immunoprecipitates from different individuals varied widely, and this was probably due to the broad intra- and interpersonal levels of hepcidin.[39] Moreover, α_2-M was confirmed as a major hepcidin-binding protein by other approaches described as shown in the next section.

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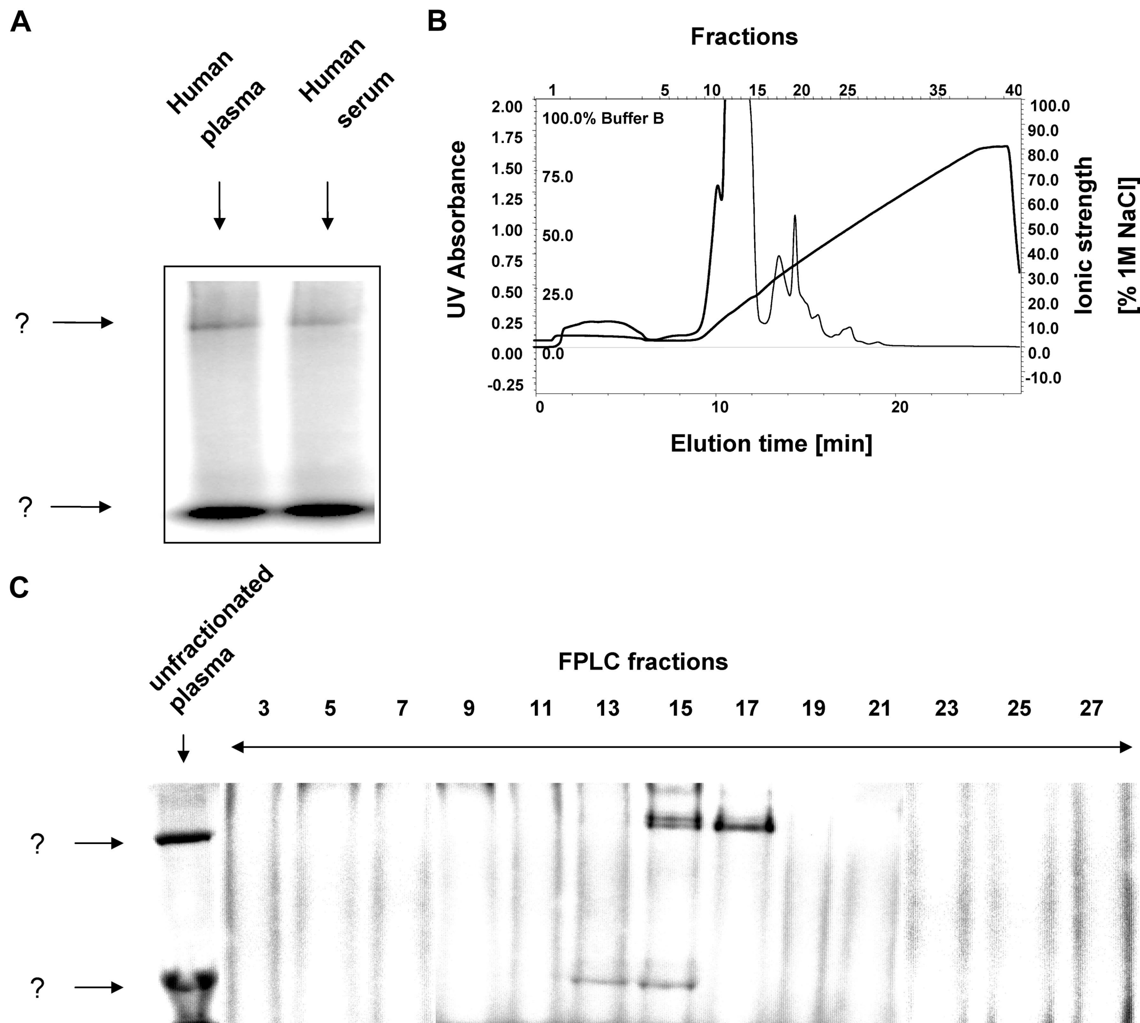

Figure 2. ^{125}I-hepcidin binds to 2 unknown molecules in plasma and serum. (A) Human plasma (10 μL) or serum (10 μL) were incubated with ^{125}I-human hepcidin (2.8 μM) for 1 hour at 37°C and 2 bands were detected (↓) corresponding to a complex of ^{125}I-human hepcidin with unknown binding proteins. (B) Native fast-pressure liquid chromatography (FPLC) fractionation of blood plasma followed by (C) separation of fractions using native-gradient PAGE. FPLC fractions were trace labeled with ^{125}I-hepcidin and separated by native gradient PAGE. After electrophoresis, the gel was vacuum dried, exposed to a storage phosphorimaging screen, and scanned on a phosphorimager. Results are typical of 3 or 4 experiments.

Super-shift analysis further confirms α_2-M is a hepcidin-binding protein

Additional evidence that α_2-M was the hepcidin-binding protein was obtained by supershift analysis (Figure 4A,B). Human plasma was incubated with ^{125}I-hepcidin (2.8 μM) for 1 hour at 37°C. This sample was then divided into equal portions to which increasing amounts of anti–α_2-M antibody was added (5-20 μL; concentration: 200 μg/mL) and the samples were separated using native-gradient PAGE. Increasing amounts of anti–α_2-M antibody led to a progressive supershift of the band into the sample well (Figure 4A,B). This was consistent with the formation of a high M_r complex between the antibody and ^{125}I-hepcidin-α_2-M complex, which, due to its large size, remained in the sample well (Figure 4A). It is relevant to note that hepcidin is a cationic peptide and thus unbound hepcidin does not migrate into the gel. Hence, in the absence of anti–α_2-M antibody (Figure 4A lane 1), some radioactivity still remained in the sample well. Control studies using a nonspecific antibody (ie, anti–cyclin D1) did not result in a supershift (Figure 4A). Collectively, these studies demonstrated that anti–α_2-M antibody super-shifts the ^{125}I-hepcidin-α_2-M complex, again confirming that α_2-M is a hepcidin-binding protein.

Size-exclusion chromatography via FPLC demonstrates α_2-M is a hepcidin-binding protein

Binding of hepcidin to α_2-M was further confirmed by size-exclusion chromatography using FPLC. As shown in the inset of Figure 5, a complex of purified α_2-M and ^{125}I-hepcidin comigrated with the peak of radioactivity formed in blood plasma after addition of ^{125}I-hepcidin. Again, this evidence supports the experiments above, confirming the role of α_2-M as a hepcidin-binding protein.

Serum albumin is the second ^{125}I-hepcidin-binding protein, but binds the peptide nonspecifically

As shown in Figure 2A and C, 2 major bands were identified as hepcidin-binding proteins at the top and bottom of the gel. The studies above clearly identified the top band as α_2-M. Identification of the ^{125}I-hepcidin-binding protein in the lower band (Figure 2 A,C) was performed in an analogous way as described above for the top band

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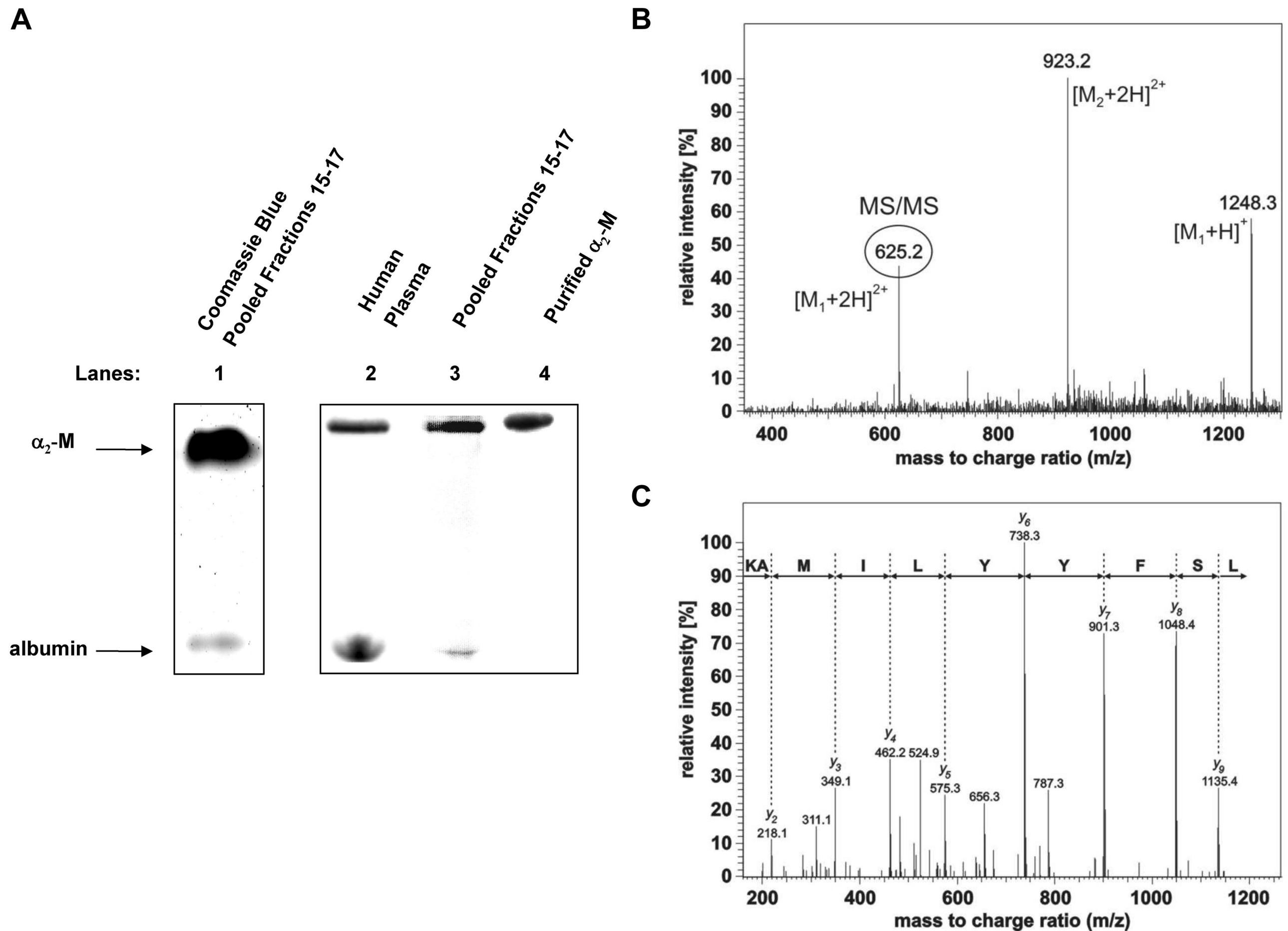

Figure 3. Identification of α_2 macroglobulin as a hepcidin-binding protein. (A) A hepcidin-binding protein in complete human plasma or fractionated plasma comigrates with a complex of purified α_2-macroglobulin (α_2-M) and ^{125}I-hepcidin. Lane 1: fractions 15 through 17 from the plasma fractionation experiment (see Figure 2C) were pooled and concentrated, separated by native gradient PAGE, and stained using Coomassie blue. Lanes 2 through 4 (phosphorimager scan of a different gel run in parallel to the Coomassie blue gel in lane 1): complete human plasma (lane 2), pooled fractions 15 through 17 (lane 3; from Figure 2C) and purified α_2-M (10 μg; lane 4) were incubated with ^{125}I-hepcidin (2.8 μM) for 1 hour at 37°C and separated by native PAGE and visualized using a phosphorimager. (B,C) Identification of the hepcidin-binding protein in blood plasma by mass spectrometry. MS/MS spectrum confirming the identity of α_2-M in the top band of panel A. The region of the gel corresponding to the top band that consists of ^{125}I-hepcidin bound to an unknown plasma protein from pooled fractions 15 through 17 (Figure 3A lane 3) was cut from the gel and digested with trypsin. The resulting tryptic peptides were separated on a C18 column connected online to the mass spectrometer. Each full scan experiment measuring the peptide masses was followed by 3 MS/MS fragmentation scans providing sequence information of the fragmented peptides. As a representative example of one peptide identification, the full MS scan (B) and fragmentation MS/MS spectrum of the peptide ^{493}LSFYYLIMAK^{502} (C) from α_2-M is shown. The MS spectrum exhibits intense signals at m/z 625.2 (circled) and 1248.3. These signals represent doubly- and singly-charged ions of the peptide, respectively. The near complete series of C-terminal y-ions in the MS/MS spectrum of doubly-charged ions clearly confirms the α_2-M peptide sequence ^{493}LSFYYLIMAK^{502} (panel C top line). The major peak in the full scan spectrum at m/z 923.2 corresponds to an additional coeluting peptide originating from α_2-M, as was confirmed by its MS/MS spectrum (not shown).

(α_2-M). The fractions 15 through 17 from the FPLC-resolved human plasma were pooled (Figure 2C), trace labeled with ^{125}I-hepcidin (20 nM), and resolved by native gradient PAGE. After this band was cut from the gel and analyzed by mass spectrometry for its protein components, albumin was found to be the hepcidin-binding protein in the lower band (Table 2 band 2). Moreover, a Coomassie blue stained band of albumin (Sigma-Aldrich) shown in Figure 6A (second lane) corresponded to the most abundant protein in plasma, namely albumin (Figure 6A first lane). The lower-migrating ^{125}I-hepcidin-binding protein in human plasma (Figure 6B first lane) comigrated with purified albumin

Table 2. Identification of the hepcidin-binding protein in plasma as α-2-macroglobulin

Band no.	Protein name	DTB no.	MW, kDa	No. of peptides
1	**α-2-macroglobulin precursor***	**P01023**	**163**	**55**
1	Ig kappa chain C region	P01834	12	2
1	Ig lambda chain C regions	P01842	11	3
1	Ig gamma-1 chain C region	P01857	36	6
1	Ig gamma-2 chain C region	P01859	36	6
1	Ig gamma-4 chain C region	P01861	36	2
1	Ig alpha-1 chain C region	P01876	38	4
2	Serum albumin precursor	P02768	69	40

DTB indicates Swiss-Prot number; and MW, molecular weight.

*α_2-M: P01023: Pooled fractions 15 through 17 (see Figure 3A lane 3) were cut from the gel, rehydrated, digested with trypsin, and subjected to MS analysis. The unknown protein was identified by LC-MS/MS analysis as α_2-M. The results are typical from 3 experiments.

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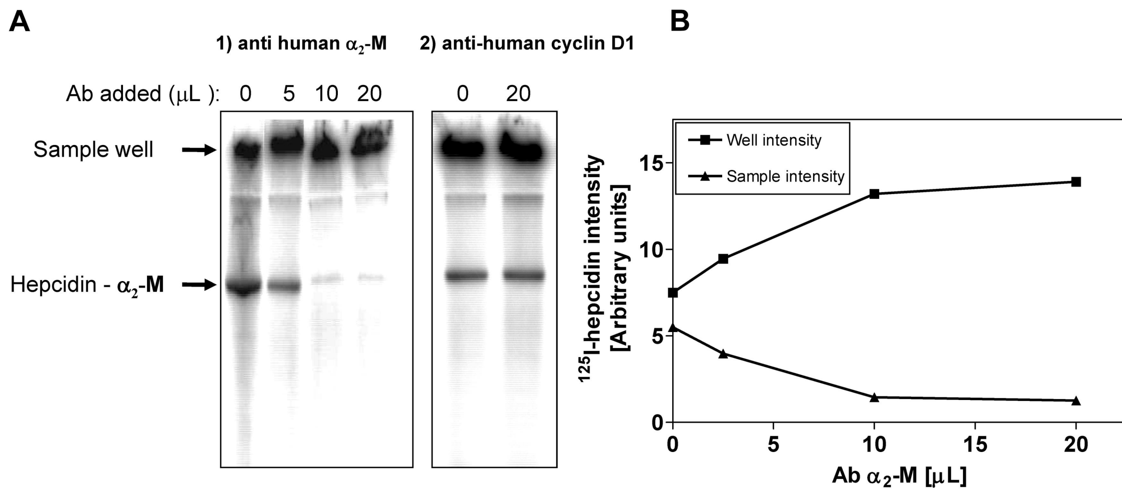

Figure 4. Hepcidin-binding protein in plasma is supershifted with an anti–α_2-M antibody. (A) Human plasma was incubated with ^{125}I-human hepcidin (2.8 μM) for 1 hour at 37°C. This sample was then divided into portions to which increasing amounts of anti–α_2-M antibody (5-20 μL; concentration 200 μg/mL) or anti–cyclin D1 antibody (20 μL; concentration 200 μg/mL) were added. The samples were separated using native-gradient PAGE. After electrophoresis, the gel was vacuum dried, scanned, and analyzed using a phosphorimager. Results are typical from 3 separate experiments. (B) The radioactivity in the ^{125}I-hepcidin–α_2-M protein band (▲) and the sample wells (■) were plotted to show that the addition of anti–α_2-M antibody progressively decreases the radioactivity in the ^{125}I-hepcidin–binding protein band and increases the activity in the sample wells. The activity in the wells is because the high molecular weight antibody–α_2-M complex cannot penetrate into the gel. The radioactivity in the sample well with no antibody is caused by the presence of cationic free hepcidin, which does not migrate into the gel. Results are typical of 3 separate experiments.

labeled with ^{125}I-hepcidin and detected by phosphorimaging (Figure 6B second lane). These experiments confirmed that albumin was the lower rapidly migrating ^{125}I-hepcidin–binding protein in Figure 2A and C.

Hepcidin-binding to albumin was found to be nonspecific, displaying nonsaturable kinetics in the range of up to a 1000-fold molar excess of hepcidin over albumin (Figure 6C). By quantitating radioactivity in the 2 hepcidin-containing bands (Figure 6B first lane), we found that 30% of plasma ^{125}I-hepcidin was bound to α_2-M, and the rest was associated with albumin. Considering this observation in relation to the fact that in humans the concentration of α_2-M (~ 2.8-5.5 μM)[40,41] is 180 to 360 times less than albumin (≈ 1 mM),[42] it was clear α_2-M is the major and specific hepcidin transport molecule. Because the concentration of human hepcidin in plasma varies from 0 to 1.5 μM,[39] it is apparent there is an excess of α_2-M. Thus, it was important to determine the affinity of α_2-M for hepcidin to assess the specificity of the interaction that is crucial for understanding its activity.

Identification of a cooperative mechanism of hepcidin binding to activated α_2-M

To determine the α_2-M-hepcidin affinity constant and stoichiometry, we examined ^{125}I-hepcidin binding to α_2-M to assess the hepcidin concentration required to saturate a constant amount of α_2-M. We could not use the measurement of free and bound ^{125}I-hepcidin because, in contrast to the ^{125}I-hepcidin-α_2-M complex, the free unbound hepcidin is a cationic peptide[13] that does not migrate into gels. To separate the α_2-M-^{125}I-hepcidin complex, we performed native electrophoresis at 37°C to provide physiologically relevant temperature, ionic strength, and pH for the determination of the hepcidin-binding constant with both α_2-M and α_2-M-MA. This is an established technique used to examine interactions of physiologic ligands with α_2-M.[43,44]

Under physiologic conditions, α_2-M is activated to bind ligands by a range of proteases.[45,46] This can be closely mimicked by

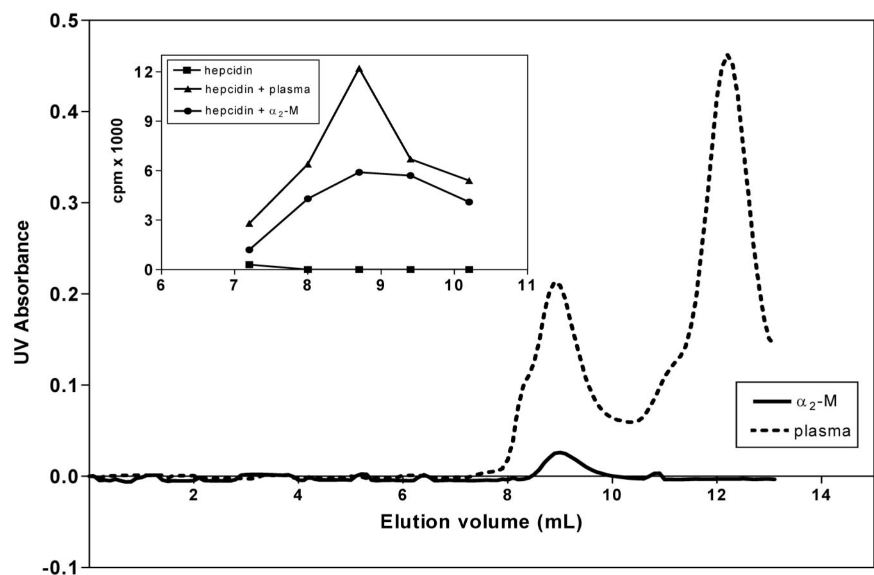

Figure 5. Analysis of the hepcidin-α_2-M interaction using native FPLC. The main graph shows the UV absorbance of fractions of human plasma (broken line) and purified α_2-M (broken line) separated by gel-size chromatography using FPLC ("Methods"). The inset shows that the complex of purified α_2-M and ^{125}I-hepcidin comigrates with the peak of radioactivity formed in blood plasma after addition of ^{125}I-hepcidin. Free ^{125}I-hepcidin (■); ^{125}I-hepcidin incubated for 1 hour at 37°C with either plasma (▲; concentration 2.8 μM) or purified α_2-M (●; 1.4 μM). Results are typical of 3 experiments.

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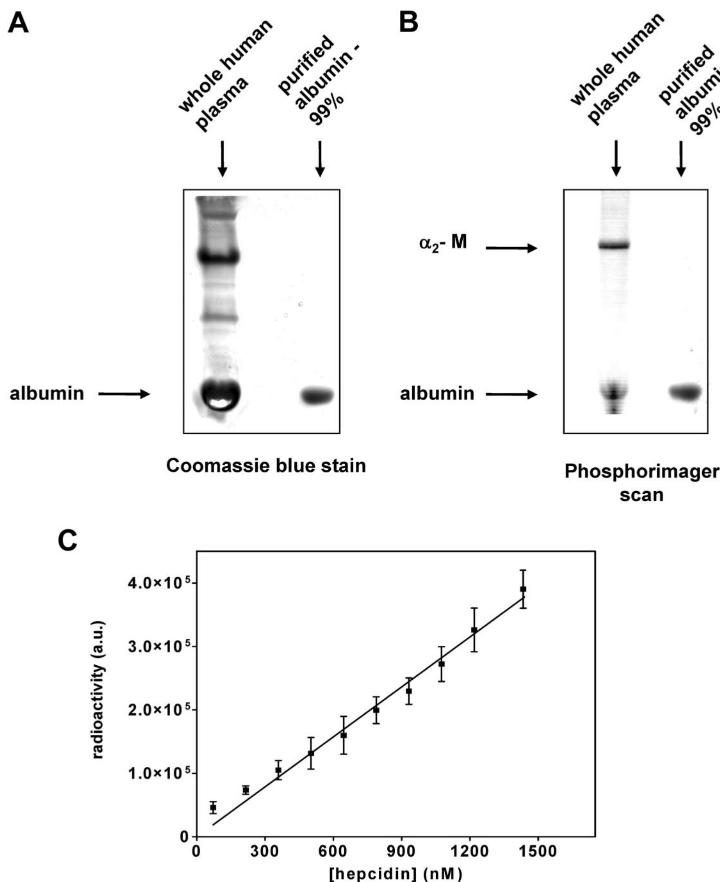

Figure 6. Confirmation that albumin is a second hepcidin-binding protein that binds this ligand nonspecifically. (A) Whole human plasma and albumin (purity: 99%) were separated using native PAGE and stained with Coomassie blue for protein. Note that the band of purified albumin comigrates with the most abundant protein present in human plasma (albumin). (B) Whole plasma and purified albumin were trace labeled with ^{125}I-hepcidin, resolved by native PAGE, and visualized using a phosphorimager. The band of purified albumin-^{125}I-hepcidin comigrates with the bottom band present in human plasma (albumin), which also binds ^{125}I-hepcidin. The top band in lane 1 is ^{125}I-hepcidin bound to α_2-M. (C) Increasing amounts of ^{125}I-hepcidin (up to 1400 nM) were added to albumin (1.5 nM), the mixture was incubated for 1 hour at 37°C, separated by native PAGE, and the albumin–^{125}I-hepcidin complex was visualized and quantified using a phosphorimager. Results in panels A and B are typical of 3 experiments while those in panel C are mean ± SD of 3 experiments.

treatment with methylamine (see "Materials"),[24] and this method is preferred to protease activation that nonspecifically damages α_2-M, perturbing the results. In fact, activation by MA is the established method of choice for examining binding of ligands to activated α_2-M.[24,27,43,44] Hence, in this investigation, we assessed the binding of hepcidin to α_2-M and its MA-activated form (α_2-M-MA; Figure 7A-C). Binding of ^{125}I-hepcidin to nonactivated α_2-M resulted in a curve indicating a single saturable class of noninteracting ^{125}I-hepcidin-binding sites (Figure 7A). Fitting of these data and Scatchard analysis led to an estimate of 2 hepcidin-binding sites per α_2-M molecule, with a K_d of 177 (± 27) nM (equation 3). Using ^{125}I-hepcidin, we were able to demonstrate that increasing concentrations of unlabeled hepcidin compete with ^{125}I-hepcidin, preventing binding of the label to α_2-M (Figure 7B). These data demonstrate that the labeled peptide was both functional and competitive at concentrations approximated by the previously measured K_d in binding with the unlabeled peptide.

In accordance with previous studies,[20] MA-activation caused a mobility shift in α_2-M migration on native electrophoresis, suggesting a conformational change (Figure 7C). In contrast to nonactivated α_2-M (Figure 7A), activated α_2-M-MA bound hepcidin and resulted in a sigmoidal saturation curve as a function of increasing ^{125}I-hepcidin concentration (Figure 7D). The shape of this curve was typical of cooperative allosteric binding[47] and its analysis using a modified Hill equation (see "Methods") demonstrated that activation of α_2-M led to the appearance of additional binding sites. Fitting to the data gave the number of binding sites as $n{\sim}3$ with a K_d for each site being approximately 0.3 μM. The estimate of $n{\sim}3$ represents the lowest number of hepcidin molecules bound to the α_2-M oligomer, as the Hill equation does not account for subtle variations between affinities of the sites, thus limiting precision in this calculation.

To definitively define the stoichiometry of hepcidin-binding to α_2-M-MA, ultracentrifugation was used (see "Methods"). This method enabled separation of bound hepcidin (pelleted) and unbound hepcidin (not pelleted) and demonstrated that each molecule of α_2-M or α_2-M-MA bound 1.98 ± 0.11 and 4.1 ± 0.19 (3 experiments) molecules of hepcidin, respectively. This value was further substantiated by the data in Figure 7D, showing that the binding curve plateaued as the hepcidin to α_2-M molar ratio was increased to 4. Collectively, our data indicate the presence of 4 hepcidin-binding sites per α_2-M-MA molecule.

The α_2-M-hepcidin complex decreases ferroportin expression in J774 cells

To assess the functional effect of the α_2-M-hepcidin complex, studies were initiated using commercial α_2-M (Sigma-Aldrich) and J744 cells that express ferroportin[48,49] and respond to hepcidin by reducing ferroportin expression.[50,51] Cells were incubated for 6 hours at 37°C in media without FCS containing: hepcidin (0.7 μM), α_2-M (2.8 μM), hepcidin (0.7 μM) plus α_2-M (2.8 μM), albumin (2.8 μM), or hepcidin (0.7 μM) plus albumin (2.8 μM; Figure 7E). Before the addition to cells, all solutions were incubated for 1 hour at 37°C to ensure complex formation. Hepcidin only slightly ($P > .05$) reduced ferroportin, while α_2-M alone or α_2-M plus hepcidin significantly ($P < .05$ and $P < .01$, respectively) decreased its expression (Figure 7E). A potential reason for α_2-M alone reducing ferroportin expression may be contamination with hepcidin present in the purified α_2-M, as found in studies examining other α_2-M ligands.[14] This effect of α_2-M

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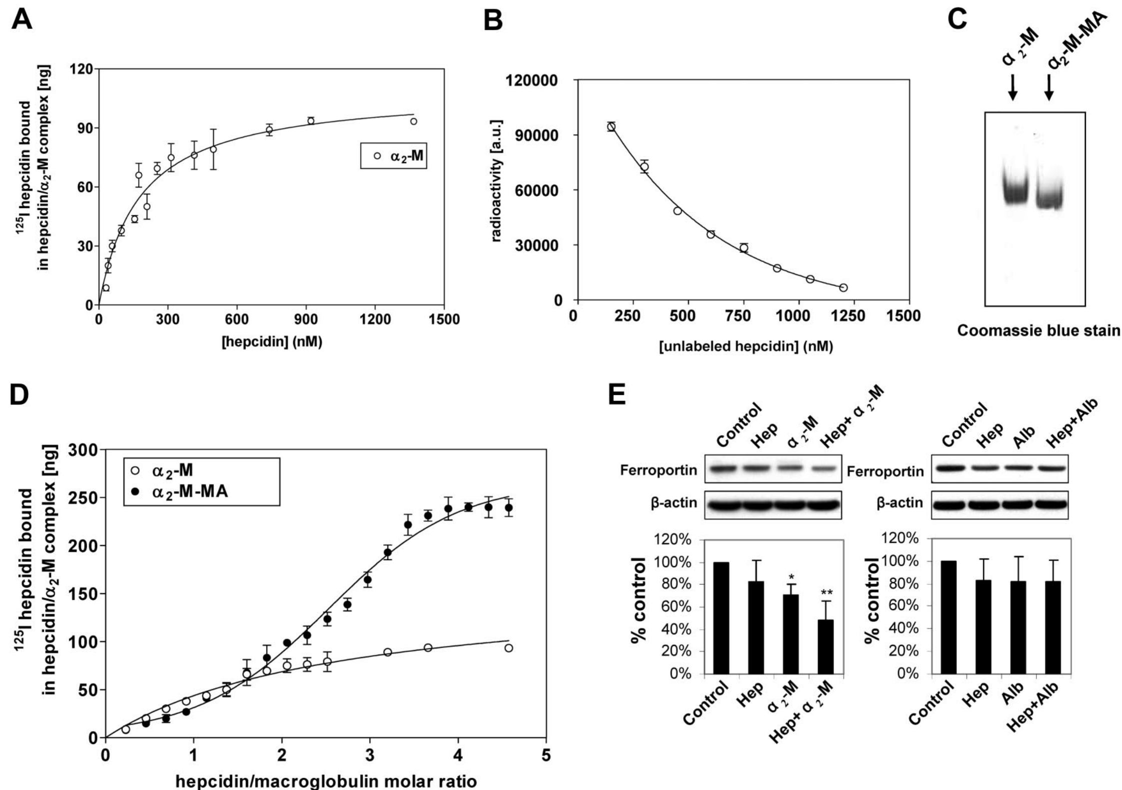

Figure 7. Characterization of α_2-macroglobulin interaction with hepcidin and confirmation of the biologic activity of the α_2-macroglobulin-hepcidin complex. (A) Saturation binding curve of ^{125}I-human hepcidin with nonactivated α_2-M demonstrating a single class of noninteracting binding sites. (B) Increasing concentrations of unlabeled hepcidin competes with ^{125}I-hepcidin preventing binding of the label to α_2-M. (C) Migration of methylamine-activated human macroglobulin (α_2-M-MA) differs from nonactivated α_2-M. (D) Saturation binding curve of ^{125}I-human hepcidin with α_2-M-MA demonstrating sigmoidal binding. This indicated allosteric cooperativity and the identification of higher order hepcidin-binding to α_2-M-MA. In contrast, nonactivated α_2-M demonstrated a hyperbolic function that corresponded to 2 independent binding sites with the same affinity. (E) Western analysis demonstrating that the α_2-M–hepcidin complex, but not the α_2-M–albumin complex reduces ferroportin expression in J774 cells. (A,D) Samples of 100 μg α_2-M or α_2-M-MA were incubated for 1 hour at 37°C with increasing concentrations of ^{125}I-human hepcidin. The samples were separated using native gradient (3%-12%) PAGE. After electrophoresis, the gel was scanned on a phosphorimager and analyzed using Aida and GraphPad software. (B) α_2-M (143 nM) was incubated for 1 hour at 37°C with ^{125}I-human hepcidin (717 nM) and increasing concentrations of unlabeled hepcidin from 150 to 1200 nM. The samples were separated using native gradient (3%-12%) PAGE. After electrophoresis, the gel was scanned and analyzed as in panels A and D. (C) α_2-M and α_2-M-MA were resolved on a native gel as described for panels A and C and then stained with Coomassie blue protein stain. (E) J774 cells were incubated for 6 hours at 37°C in culture media without FCS containing either hepcidin (0.7 μM), α_2-M (2.8 μM), hepcidin (0.7 μM) + α_2-M (2.8 μM), albumin (2.8 μM), or hepcidin (0.7 μM) + albumin (2.8 μM). Results in panels A, B, and D are mean ± SD from 3 separate experiments, while panel C shows a typical gel from 3 experiments performed. The Western analysis shown in panel E is from a typical blot, while the densitometric scan is mean ± SD from 5 or 6 experiments. $^*P < .05$; $^{**}P < .01$.

alone was observed in different lots of the protein from Sigma-Aldrich and also when it was purchased from a different source (Sapphire Bioscience, Sydney, Australia). In contrast to the α_2-M plus hepcidin treatment, albumin plus hepcidin did not lead to any significant decrease in ferroportin (Figure 7E). These experiments demonstrate that the α_2-M-hepcidin complex is functionally active and more effective than hepcidin alone at reducing ferroportin expression. Furthermore, it should be noted that this is not a nonspecific effect, as hepcidin added to the protein control (albumin) at the same concentration as α_2-M had no significant effect on ferroportin expression. Previous studies by Kaplan and colleagues[1] have shown that the effect of hepcidin on down-regulating ferroportin is far more complete than that demonstrated in Figure 7E. However, it is notable that Kaplan et al used HEK293 cells transfected with ferroportin, which hyperexpress this protein.[1] Hence, the comparison to J744 cells cannot be readily made as the latter express ferroportin under a physiologic control mechanism. Furthermore, the study of Kaplan et al assessed membrane ferroportin expression, while we examined total cellular ferroportin, which is less likely to demonstrate ablation after only 6 hours of incubation with α_2-M–hepcidin. Other authors using western blots have also shown, in J774 cells, that the response of ferroportin to hepcidin is less than complete.[50]

Discussion

We hypothesized that in analogy to other hormones,[52-54] hepcidin would circulate bound to specific binding proteins. We used ^{125}I-hepcidin to demonstrate the presence of hepcidin-binding proteins in human blood. To identify these proteins by mass spectrometry, we purified ^{125}I-labeled hepcidin-binding protein complexes using a native 2-dimensional technique consisting of FPLC and nondenaturing PAGE.[17] The hepcidin-binding proteins were identified as α_2-M and albumin (Figures 3-6).

The identity of α_2-M as a specific, high-affinity, hepcidin-binding protein was confirmed by the following evidence: (1) purified α_2-M binds ^{125}I-hepcidin and this complex comigrates with the band obtained by adding ^{125}I-hepcidin to fractionated or whole plasma as shown in Figure 3A; (2) the hepcidin-binding protein complex in plasma was recognized and super-shifted with an anti–α_2-M antibody (Figure 4), but not by a nonspecific control antibody; (3) native size-exclusion chromatography demonstrated the ^{125}I-hepcidin-carrier protein complex in plasma was coeluting with ^{125}I-hepcidin added to purified α_2-M (Figure 5); and (4) K_d measurements demonstrated specific ^{125}I-hepcidin-binding to 2 (α_2-M)

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and 4 (α_2-M-MA) high-affinity sites on the protein, with evidence of allosteric cooperativity in α_2-M-MA (Figure 7). These studies were confirmed by ultra-centrifugation, where α_2-M or α_2-M-MA bound 2 or 4 hepcidin ligands per molecule, respectively.

The current results are significant, as it has been previously thought that due to the low M_r of hepcidin ($\approx$ 2800 Da),[55] it would be rapidly cleared by the kidney, reducing its half-life. The specific binding of α_2-M prevents this clearance, and, hence, markedly modulates the activity of the peptide. If we consider a theoretical calculation where all α_2-M is activated (K_d: 0.3 μM) and the affinity of albumin for hepcidin is weak (assumed/approximated K_d:1 mM), then approximately 11% of hepcidin would be free. This may explain the presence of some hepcidin in urine.[13]

This is the first demonstration that α_2-M is a high-affinity hepcidin-binding molecule. Moreover, upon activation, there is evidence of multiple allosteric binding sites on α_2-M-MA, and this has not been described for its other ligands. Further, these observations suggest a regulatory role for α_2-M on hepcidin function. It is known that α_2-M is a multifunctional plasma protein.[24] The first described function of α_2-M was its "trapping" and inhibition of proteases.[56] This inhibition process triggers a conformational change in α_2-M, converting it to the activated form, which binds hormones, signaling molecules, etc.[24] In fact, numerous molecules bind to α_2-M and α_2-M-MA, such as transforming growth factor-β types 1 and 2,[57,58] platelet-derived growth factor,[59] nerve growth factor,[60] tumor necrosis factor-α,[15] basic fibroblast growth factor,[61] interleukins-1, -6, and -8,[62-64] vascular endothelial growth factor,[65] growth hormone,[66] osteoprotegerin,[14] leptin,[52] activin, and inhibin.[67] The binding affinities (K_d) of α_2-M and α_2-M-MA for their ligands are rarely described, but for some[24,68,69] it was reported to lie within 10^{-7} to 10^{-9} M, which corresponds to the affinity of α_2-M for hepcidin (ie, α_2-M, K_d = 177 nM; α_2-M-MA, $K_d \approx$ 0.3 μM) observed herein.

Panyutich and Ganz[36] demonstrated that α_2-M-MA binds antimicrobial peptides known as defensins, which have some homology to hepcidin. However, these authors did not demonstrate cooperative allosteric binding. Considering the cooperativity found upon hepcidin-binding to α_2-M-MA (Figure 7D), it is relevant to discuss that a sigmoidal binding curve is also observed when O_2 binds with the 4 heme centers of hemoglobin, leading to efficient uptake and release.[47] Analogously, the efficient binding and release of 4 hepcidin molecules to α_2-M mediated via allosteric activation may be crucial for its function. It is notable that again, like hemoglobin, α_2-M is composed of 4 subunits,[24] thus alterations in interactions between subunits may lead to cooperative binding.

The functionality of the α_2-M–hepcidin complex was shown in our studies where it was more effective than hepcidin alone at reducing ferroportin-1 expression (Figure 7E). The precise mechanism inducing this effect is unknown. However, ligands bound to α_2-M undergo endocytosis by binding to the α_2-M receptor.[24,70,71] This receptor is found in many cell types,[72] and mediates ligand uptake and delivery to endosomes and lysosomes.[24,73,74] Therefore, considering the ability of α_2-M to target its ligands to cells,[24] it is conceivable that hepcidin-binding to α_2-M could influence hepcidin activity. Such alterations may include the half-life of hepcidin, its tissue transfer, and the binding of hepcidin to ferroportin. It is also possible that the α_2-M–hepcidin complex binds to other receptors. Finally, the hepcidin-MG complex could play some signaling role once it is processed by the lysosome.

In conclusion, identification of α_2-M as the hepcidin carrier will lead to deeper understanding of its role in iron metabolism. Because hepcidin has diagnostic potential, any method for determination of its blood concentration must take into account hepcidin-binding proteins. This investigation is important for understanding hepcidin function and its use in diagnostic tests.

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Authorship

Contribution: G.P., J.P., K.K., I.H., P.H., P.W.K., S.S.-L., P.P., R.S., E.B., M.L.-H.H., and Y.S.R. designed and performed experiments and wrote sections of the manuscript; and D.V. and D.R.R. designed the study, obtained grant funding, and wrote the manuscript.

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