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Hepcidin Bound to α_2-Macroglobulin Reduces Ferroportin-1 Expression and Enhances Its Activity at Reducing Serum Iron Levels

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Michael Li-Hsuan Huang[‡], Christopher J. D. Austin[‡], Marie-Agnès Sari[§], Yohan Suryo Rahmanto[‡], Prem Ponka[¶1], Daniel Vyoral[‖2], and Des R. Richardson[‡1,3]**

*From the [‡]Department of Pathology, University of Sydney, Sydney, New South Wales 2006, Australia, the [§]Université Paris Descartes, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR8601 CNRS, 45 Rue des Saints Peres, 75006 Paris, France, the [¶]Lady Davis Institute for Medical Research, Montreal, Quebec H3T1E2, Canada, the [‖]Institute of Hematology and Blood Transfusion, U Nemocnice 1, Prague 2, 128 20, Czech Republic, and the **First Faculty of Medicine, Institute of Pathological Physiology, Charles University in Prague, U Nemocnice 5, Prague 2, 128 53, Czech Republic*

Background: Hepcidin is the hormone of iron metabolism that is bound by α_2-macroglobulin (α_2M) and its activated counterpart (α_2M-MA).
Results: Serum iron is reduced to a greater extent in mice treated with α_2M·hepcidin or α_2M-MA·hepcidin relative to unbound hepcidin.
Conclusion: α_2M retards hepcidin excretion by the kidney, increasing its efficacy.
Significance: These results are important for understanding hepcidin transport and detection in blood.

Hepcidin regulates iron metabolism by down-regulating ferroportin-1 (Fpn1). We demonstrated that hepcidin is complexed to the blood transport protein, α_2-macroglobulin (α_2M) (Peslova, G., Petrak, J., Kuzelova, K., Hrdy, I., Halada, P., Kuchel, P. W., Soe-Lin, S., Ponka, P., Sutak, R., Becker, E., Huang, M. L., Suryo Rahmanto, Y., Richardson, D. R., and Vyoral, D. (2009) *Blood* 113, 6225–6236). However, nothing is known about the mechanism of hepcidin binding to α_2M or the effects of the α_2M·hepcidin complex *in vivo*. We show that decreased Fpn1 expression can be mediated by hepcidin bound to native α_2M and also, for the first time, hepcidin bound to methylamine-activated α_2M (α_2M-MA). Passage of high molecular weight α_2M·hepcidin or α_2M-MA·hepcidin complexes (≈725 kDa) through a Sephadex G-25 size exclusion column retained their ability to decrease Fpn1 expression. Further studies using ultrafiltration indicated that hepcidin binding to α_2M and α_2M-MA was labile, resulting in some release from the protein, and this may explain its urinary excretion. To determine whether α_2M-MA·hepcidin is delivered to cells via the α_2M receptor (Lrp1), we assessed α_2M uptake and Fpn1 expression in *Lrp1*$^{-/-}$ and *Lrp1*$^{+/+}$ cells. Interestingly, α_2M·hepcidin or α_2M-MA·hepcidin demonstrated similar activities at decreasing Fpn1 expression in *Lrp1*$^{-/-}$ and *Lrp1*$^{+/+}$ cells, indicating that Lrp1 is not essential for Fpn1 regulation. *In vivo*, hepcidin bound to α_2M or α_2M-MA did not affect plasma clearance of α_2M/α_2M-MA. However, serum iron levels were reduced to a significantly greater extent in mice treated with α_2M·hepcidin or α_2M-MA·hepcidin relative to unbound hepcidin. This effect could be mediated by the ability of α_2M or α_2M-MA to retard kidney filtration of bound hepcidin, increasing its half-life. A model is proposed that suggests that unlike proteases, which are irreversibly bound to activated α_2M, hepcidin remains labile and available to down-regulate Fpn1.

Hepcidin plays a crucial role in regulating iron metabolism (1, 2) and was initially discovered in urine (2) and serum (3). This disulfide-rich peptide is synthesized in the liver and then transported in blood to its target cells, *e.g.* enterocytes, macrophages, hepatocytes, etc. (1). In these cell types, hepcidin regulates the trans-membrane transport of iron through interaction with ferroportin-1 (Fpn1),[4] which is responsible for iron efflux (4). Once hepcidin binds to Fpn1, the complex becomes internalized and degraded within the lysosome (5). This results in a decrease in the effective number of iron exporters on the plasma membrane, thereby reducing iron release from cellular stores (6).

The transport of hepcidin within the blood is of interest as: 1) many peptide hormones are bound by carriers that influence their function and distribution (7–13); 2) a greater understanding of the mechanism of hepcidin delivery to the cell could provide further information on its regulatory role; and 3) understanding the binding of hepcidin to a carrier protein would facilitate development of quantitative hepcidin assays.

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[3] Supported by Senior Principal Research Fellowship 571123 and Project Grant 632698 from the National Health and Medical Research Council of Australia. To whom correspondence should be addressed: Dept. of Pathology, Blackburn Bldg., D06, University of Sydney, NSW 2006, Australia. Tel.: 61-2-9036-6548; Fax: 61-2-9351-3429; E-mail: d.richardson@med.usyd.edu.au.

[4] The abbreviations used are: Fpn1, ferroportin-1; α_2M, α_2-macroglobulin; α_2M-MA, methylamine-activated α_2-macroglobulin; Lrp1, low density lipoprotein receptor-related protein-1; HNP1, defensin; DFO, desferrioxamine; FAC, ferric ammonium citrate; MEF, mouse embryonic fibroblast; IAA, iodoacetamide; CON, control; BioHepcidin, biotinylated hepcidin.

We discovered that hepcidin preferentially binds to the blood glycoprotein, α_2-macroglobulin (α_2M) (14), and this observation was subsequently and independently confirmed by two other groups (15, 16). Significantly, Ganz and co-workers (17) also showed that the peptide, defensin (HNP1), which belongs to the same family of highly disulfide-bonded peptides, also binds to α_2M. Nonetheless, a very preliminary study has indicated that α_2M binds only a small fraction of the total hepcidin in human blood (16). However, in this latter report, it was unclear what type of α_2M (native, activated or ligand-bound) was implemented to calibrate the gel chromatography column used to isolate α_2M (16). This point is crucial, as α_2M elutes in different chromatographic fractions depending on its form. Hence, although it was evident that α_2M was identified in a fraction (16), it was unclear what proportion of total α_2M it represented. Therefore, the analysis performed was questionable and did not agree with two other independent studies (14, 15) and the investigation herein.

It is known that the activity of α_2M in inhibiting proteases is mediated via protease binding to a "bait region" of α_2M, which results in a conformational change to its active form (18), and this can be mimicked by methylamine treatment (14, 19, 20). Activated α_2M is rapidly internalized by endocytosis via binding to low density lipoprotein receptor-related protein-1 (Lrp1) (18). Notably, the ligand binding activity of α_2M is mediated by several binding sites, not only the bait region (13, 21–23).

Our previous investigation demonstrated that hepcidin binding to native α_2M displays high affinity (K_d 177 ± 27 nM), and this leads to down-regulation of Fpn1 (14). Moreover, hepcidin binds to the methylamine-activated form of α_2M (α_2M-MA) via four high affinity (K_d 300 nM) binding sites (14). The ability of α_2M-MA-bound hepcidin to down-regulate Fpn1 is unknown and is examined in this study.

In addition to hepcidin binding, both α_2M and α_2M-MA are known to bind many hormones and/or cytokines *e.g.* defensin (HNP1) (17), transforming growth factor-β1/2, etc. (7–13, 24). The consequence of these interactions with α_2M/α_2M-MA can be passive (24) or stimulatory (10). However, the effects of binding to α_2M/α_2M-MA can also be inhibitory (9, 11–13) via the prevention of hormone-receptor binding (9, 13, 25). This effect can also differ between α_2M and α_2M-MA (10). Hence, in this study, we have assessed the binding of hepcidin to α_2M and α_2M-MA and its downstream effects on its target Fpn1.

In this study, we demonstrate that the complexes formed from the binding of hepcidin to α_2M or α_2M-MA elicit a decrease in Fpn1 expression in the J774 murine macrophage cell line. This cell type was used because macrophages play key roles in cellular iron metabolism via recycling iron and releasing it back to the blood (1, 26). Moreover, this cell type has been well characterized to express Fpn1 and respond to hepcidin (26, 27). Interestingly, the decrease in Fpn1 expression elicited by α_2M·hepcidin and α_2M-MA·hepcidin complexes was shown to be independent of the α_2M receptor, Lrp1, using 2-cell models and different methods. We also show that serum iron levels are reduced to a significantly greater extent in mice treated with α_2M·hepcidin or α_2M-MA·hepcidin complexes relative to unbound hepcidin. Our data indicate that α_2M acts as a hepcidin carrier, which retards its excretion by the kidney, increasing its efficacy relative to low molecular weight unbound hepcidin. We propose a model that explains hepcidin delivery to Fpn1 and also its presence in urine that is mediated via the labile binding of hepcidin to α_2M or α_2M-MA.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Human α_2M (≥98% purity) was from Sigma. Synthetic bioactive human hepcidin (25 amino acids) was from Peptides International (4392-S; Louisville, KY). Our studies showed this peptide was folded correctly as it markedly decreased the expression of its target (Fpn1) in J774 cells (see Fig. 1). Biotinylated human hepcidin (BioHepcidin; biotin attached to the lysine residue) was prepared as described previously (15). Human ^{125}I-labeled hepcidin was from Bachem (Torrance, CA) and shown to react with anti-hepcidin antibodies (company technical data sheet). All forms of hepcidin were shown to 1) bind α_2M and α_2M-MA, 2) reduce Fpn1 expression in J774 cells, and/or 3) reduce serum iron levels in mice. Methylamine, desferrioxamine (DFO), and ferric ammonium citrate (FAC) were from Sigma. Sephadex G-25 chromatography/gel filtration columns (referred to as Sephadex G-25 columns) were from GE Healthcare. Separation of unbound hepcidin from α_2M-MA·hepcidin mixtures was performed using a 5-kDa molecular mass cutoff centrifugal ultrafilter (Ultrafree-MC, Millipore, Bedford, MA).

Activation of α_2M by Methylamine—Conversion of native α_2M into its activated form (α_2M-MA) was achieved by standard methods using methylamine (14, 19, 20). Briefly, activation of α_2M was performed by incubation with 200 mM methylamine, 0.05 M Tris-HCl (pH 8.1) for 4 h at room temperature (28). Unreacted methylamine was removed from α_2M-MA using a Sephadex G-25 column. Activation of α_2M was confirmed by its mobility shift from the "slow" (native) to "fast" (activated) forms when resolved by native PAGE (14). In all instances in this study, α_2M-MA was prepared from the same lot as the α_2M used.

Cell Culture—The J774 murine macrophage-like cell line and murine embryonic fibroblast (MEF) wild-type ($Lrp1^{+/+}$) and Lrp1-null ($Lrp1^{-/-}$) cell types were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown by standard methods (14) and used at 90% confluence to maximize Fpn1 detection.

Cell Treatments—Modulation of cellular iron concentrations was achieved using established methods by incubating cells in serum-containing media supplemented with either 100 μM DFO or 250 μg/ml FAC for 24 h at 37 °C, which are known to deplete or load cells with iron, respectively (29, 30).

Studies involving hepcidin treatment and Fpn1 expression were performed in two stages. In the first stage, cells were pretreated with culture media alone (DMEM + 10% fetal calf serum; control) or this medium containing DFO or FAC for 24 h at 37 °C (primary incubation). This medium was then removed, and the cells were rinsed with serum-free media. In the second stage, cells were incubated with serum-free DMEM or this medium containing DFO (100 μM) or FAC (250 μg/ml) in the presence or absence of either hepcidin (0.7 μM), α_2M (2.8 μM), α_2M-MA (2.8 μM), or α_2M·hepcidin and α_2M-MA·hepcidin complexes (2.8 μM α_2M or α_2M-MA, 0.7 μM hep-

cidin; 4:1 ratio) for 6 h at 37 °C (secondary incubation). As DFO-treated cells demonstrate strongly suppressed Fpn1 expression due to induction of iron depletion (26), these cells did not receive subsequent treatment with hepcidin, α_2M etc., because any effect on Fpn1 expression would not be apparent. Therefore, Fpn1 expression in DFO-treated cells was used as a negative control. In some experiments, endogenous Lrp1 expression of J774 cells was down-regulated by preincubation with α_2M-MA (2.8 μM) for 24 h at 37 °C.

Western Analysis—Western analysis was performed by established methods using whole cell lysates (14). The primary antibodies used were anti-Fpn1 (MTP11A; 1:500; Alpha Diagnostics International, St. Antonio, TX), anti-Lrp1 (SC-16168; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (A5441; 1:10,000; Sigma). Secondary antibodies were anti-rabbit, anti-goat, and anti-mouse (A0545, A5420, and A9917, respectively; 1:10,000; Sigma).

Membranes were washed and developed using ECL+ Western blot detection reagent (Amersham Biosciences) and exposed to x-ray film. Densitometry was performed using Quantity One software (Bio-Rad). All data were normalized to the loading control, β-actin. Chemiluminescent immunodetection by x-ray film demonstrated a linear signal output within 5% error over the protein loading employed and the exposure times assessed. This was confirmed by digital imaging (ChemiDoc MP System, Bio-Rad), showing that the exposures were within the linear range of the film and that chemiluminescent substrate was not limiting.

Complexation of Hepcidin by α_2M or α_2M-MA and Subsequent Size Exclusion Filtration—Hepcidin was complexed with α_2M or α_2M-MA using a 1-h incubation at 37 °C, at a final concentration of 2.8 μM α_2M or α_2M-MA to 0.7 μM hepcidin (14). This 4-fold molar excess of α_2M or α_2M-MA was used throughout to ensure the majority of hepcidin was incorporated into the protein, which binds two and four molecules of hepcidin (14), respectively. Hence, for all conditions, the concentration of hepcidin used remained constant at 0.7 μM to allow direct comparison between the unbound and protein-bound forms.

To remove any unbound hepcidin, the α_2M·hepcidin/α_2M-MA·hepcidin complexes were loaded onto Sephadex G-25 columns (exclusion limit, 5 kDa) and centrifuged at 1000 × *g* for 2 min at room temperature, as per the manufacturer's instructions. As a control, 0.7 μM unbound hepcidin was also centrifuged through the Sephadex G-25 column, and the eluate was collected and then added to cells to assess its effects on Fpn1 expression.

To further ascertain if hepcidin remained complexed to α_2M-MA subsequent to Sephadex G-25 gel filtration, the α_2M-MA·hepcidin complex was loaded onto the Millipore centrifugal ultrafilter described above (cutoff, 5 kDa) and centrifuged at 5000 × *g* for 30 min at 4 °C. As controls, 0.7 μM unbound hepcidin and 2.8 μM α_2M-MA were treated similarly. The retained and eluted fractions were collected and added to the cells to test their effects on Fpn1 expression via Western analysis.

Characterization of Hepcidin Binding to α_2M or α_2M-MA—To determine the specific nature of the interaction, BioHepcidin was incubated at a 2:1 molar ratio of BioHepcidin/α_2M and a 4:1 molar ratio of BioHepcidin/α_2M-MA. In these studies, 14 pmol of α_2M or α_2M-MA were incubated with BioHepcidin for 1 h at 37 °C. Any unbound BioHepcidin was removed from the α_2M·BioHepcidin/α_2M-MA·BioHepcidin complexes using Sephadex G-25 columns, as described above. For dithiothreitol (DTT) experiments, α_2M·BioHepcidin/α_2M-MA·BioHepcidin complexes were incubated with 2 mM DTT at 100 °C for 10 min before Sephadex G-25 column desalting (17). For experiments using iodoacetamide (IAA), α_2M/α_2M-MA was incubated with 2 mM IAA at 22 °C for 1 h, followed by Sephadex G-25 column desalting and BioHepcidin incubation, as described above (31).

For experiments examining the lability of hepcidin binding at various pH values, α_2M/α_2M-MA (or these proteins treated with IAA as described above) were dissolved and incubated in either of the following buffers in 0.14 M NaCl for 30 min at 37 °C: pH 5 (sodium acetate/acetic acid buffer); pH 6 (Na_2HPO_4/NaH_2PO_4 buffer); pH 7–9 (Tris buffer); and pH 10 (sodium carbonate/sodium bicarbonate buffer). BioHepcidin was then added to the α_2M/α_2M-MA protein, as described above.

Following treatment, samples were concentrated using a vacuum concentrator (Thermo Scientific) and applied dropwise to a nitrocellulose membrane before biotin-streptavidin-HRP detection via chemiluminescence (Chemiluminescence Detection Module, Thermo Scientific). Preliminary studies showed that the biotin label did not interfere with hepcidin binding to α_2M or α_2M-MA or its ability to decrease Fpn1 expression in J774 cells (data not shown).

Radiolabeling of α_2M/α_2M-MA and Cellular Uptake Experiments—Subsequent to radioiodination, α_2M was activated by methylamine to generate α_2M-MA, as described above. The α_2M or α_2M-MA (14 nmol) was radiolabeled (32) with ^{125}I, and free ^{125}I was removed using a Sephadex G-25 column. Protein-free ^{125}I was determined by precipitation using 7.5% trichloroacetic acid (30, 33, 34) and was <0.5% of the total.

The *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ cells were incubated with 40 nM of ^{125}I-α_2M and ^{125}I-α_2M-MA, or these proteins at this concentration complexed with 10 nM hepcidin (14). Cells were incubated with radiolabeled α_2M·hepcidin/α_2M-MA·hepcidin complexes for 1 h at 37 °C to allow their uptake. The cells were then placed on ice and washed four times with ice-cold PBS. Membrane and internalized ^{125}I-α_2M or ^{125}I-α_2M-MA uptakes were assayed by standard methods using Pronase (Sigma; 1 mg/ml) for 30 min at 4 °C (30, 34, 35). Cells were removed from plates using a spatula in 1 ml of ice-cold PBS, and the cell suspension was centrifuged at 14,000 rpm for 1 min at 4 °C. The supernatant (containing the membrane-associated fraction) was separated from the cell pellet (comprising the internalized fraction) and placed into γ-counting tubes. Fractions were analyzed by a WIZARD Gamma Counter (PerkinElmer Life Sciences).

Plasma Clearance, Urinary Excretion, and Organ Distribution Studies—All animal studies were performed in 6-week-old female C57BL/6 mice raised on a standard diet. Mice were bred and handled using an approved protocol from the University of Sydney Animal Ethics Committee. To examine ^{125}I-α_2M/^{125}I-α_2M-MA metabolism in the presence or absence of hepcidin, 100 μl of 0.9% saline (vehicle) or this vehicle containing Na^{125}I

(2.5 μCi), ^{125}I-α_2M, ^{125}I-α_2M-MA, ^{125}I-α_2M·hepcidin, or ^{125}I-α_2M-MA·hepcidin (proteins, 0.36 nmol; hepcidin, 0.09 nmol; complexed at a 4:1 ratio) was injected into the tail vein. Within 10 s of injection, an aliquot (5 μl) of blood was withdrawn from a tail snip, and this measurement was taken to represent 100% of total circulating radiation (8, 36, 37). Blood samples (5 μl) were collected at predetermined times (1–60 min) in Terumo Capiject® Micro-Collection tubes (Somerset, NJ), and the radioactivity was determined using the gamma counter above.

In a separate cohort of mice, 100 μl of 0.9% saline (vehicle) or this vehicle containing Na^{125}I (2.5 μCi), ^{125}I-hepcidin, ^{125}I-hepcidin-α_2M, or ^{125}I-hepcidin-α_2M-MA (proteins, 0.36 nmol; ^{125}I-hepcidin, 0.09 nmol; complexed at a 4:1 ratio) were injected into the tail vein to examine ^{125}I-hepcidin metabolism in the presence and absence of α_2M/α_2M-MA. To examine urine excretion of ^{125}I-hepcidin, mice injected with the agents above were housed individually in cages lined with absorbent paper towels (Kimberly-Clark, Sydney, Australia) that were secured to the bottom of the cage by overlying fly-screen mesh. The radioactivity in the excreted urine was assessed at 1, 2, 4, and 24 h after injection. At each time point, the absorbent paper towel containing radioactive urine was removed and replaced with fresh paper. The radioactivity in the used paper towel was determined using the gamma counter described above. At the terminating time point (*i.e.* 24 h after injection), mice were euthanized, and major organs were collected, washed in PBS, and weighed, and the radioactivity was assessed.

Serum Iron Studies—After mice were given isofluorane anesthesia, 100 μl of 0.9% saline (vehicle) or this vehicle containing α_2M, α_2M-MA, α_2M·hepcidin, or α_2M-MA·hepcidin (proteins, 3.6 nmol; hepcidin, 0.9 nmol; complexed at a 4:1 ratio) was injected into the tail vein. After 2 h, blood (600 μl) was collected by cardiac puncture from anesthetized mice and placed in Capiject® Micro Collection tubes, and serum was isolated. Serum iron was measured using a Konelab Clinical Chemistry Analyzer (Thermo Scientific, Waltham, MA).

Statistical Analysis—Results were expressed as mean ± S.D. or S.E. Data were compared using Student's *t* test. Results were considered significant when $p < 0.05$.

RESULTS

Hepcidin Reduces Fpn1 Expression—Western blot analysis of J774 lysates detected two major protein bands with an antibody targeting the Fpn1 C terminus (Fig. 1*A*). The upper band (band I) migrated at ~62 kDa, which is the expected molecular weight of Fpn1 (38), whereas the lower band (band II) was at ~55 kDa (Fig. 1*A*). Detection of these bands has previously been described with this Fpn1 antibody (39), as well as with antibodies raised against different Fpn1 peptides (27, 40–43). These studies suggested the lower molecular weight band corresponds to Fpn1 isoforms/products derived from post-translational modification(s), such as protein cleavage (27, 39–43). In fact, in some blots, the lower band II appeared as a doublet suggesting multiple isoforms. Herein, our analysis will focus on band I (62 kDa), as it is very similar to the predicted molecular weight of Fpn1 (62.5 kDa) (38) and has been considered representative of Fpn1 expression by a number of research groups (27, 39–42).

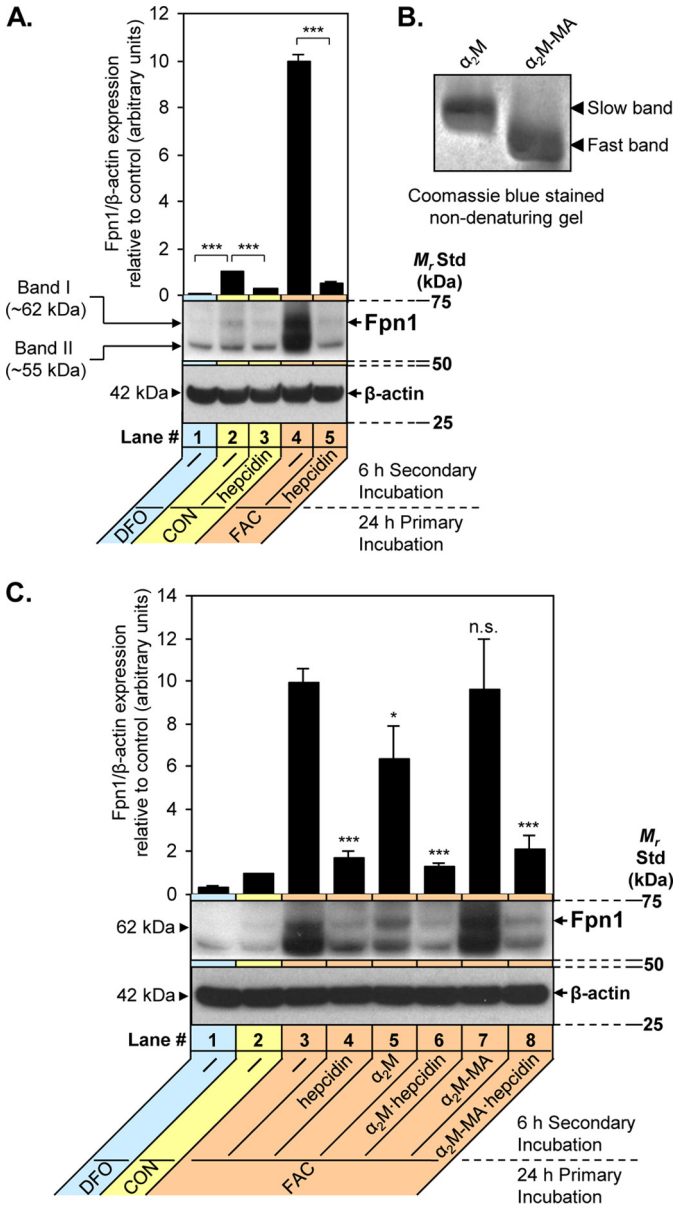

FIGURE 1. **Fpn1 is regulated by cellular iron levels, and hepcidin complexed to both native (α_2M) and activated α_2-macroglobulin (α_2M-MA) elicits similar Fpn1 reduction activity as unbound hepcidin.** *A*, J774 cells were preincubated with control (*CON*) medium or this medium containing DFO (100 μM) or FAC (250 μg/ml) for 24 h at 37 °C (primary incubation). These cells were then incubated for 6 h at 37 °C (secondary incubation) in serum-free media (namely CON, DFO, or FAC) with or without 0.7 μM hepcidin, as indicated. *B*, migration of α_2M-MA differs from native α_2M when resolved using native gel electrophoresis and stained with Coomassie Blue. *C*, J774 cells were incubated for 24 h (primary incubation) as in *A* to modulate cellular iron levels and then incubated for 6 h at 37 °C (secondary incubation) in serum-free media (namely CON, DFO or FAC) with or without hepcidin (0.7 μM), α_2M (2.8 μM), α_2M·hepcidin (2.8 μM α_2M, 0.7 μM hepcidin), α_2M-MA (2.8 μM), or α_2M-MA·hepcidin (2.8 μM α_2M-MA, 0.7 μM hepcidin). The Western blots in *A* and *C* are from typical blots from three experiments, and the densitometric values are mean ± S.D. (three experiments). The native gel in *B* is typical from three experiments. *n.s.*, $p > 0.05$; *, $p < 0.05$; ***, $p < 0.001$ relative to *lane 3* (FAC-treated cells).

As a positive control for iron depletion, J774 cells were incubated with serum-supplemented media containing the iron chelator, DFO (100 μM), for 24 h (*primary incubation*; Fig. 1*A*, *lane 1*), and then the medium was removed and replaced with serum-free media containing the same DFO concentration for a

further 6 h (*secondary incubation*). These DFO-treated cells were incubated only in the absence of hepcidin, as Fpn1 expression (62-kDa band) was significantly ($p < 0.001$) depressed relative to cells incubated with control (CON) medium only (Fig. 1*A, cf. lanes 1* and *2*). To ascertain the effectiveness of unbound hepcidin at decreasing endogenous mouse Fpn1, J774 cells were preincubated for 24 h (primary incubation) with either serum-supplemented CON medium or this medium containing the cellular iron donor, FAC (250 μg/ml), to enhance Fpn1 expression (26). After this incubation, the medium was replaced with either serum-free CON media (*lanes 2* and *3*) or this medium containing FAC (*lanes 4* and *5*) with or without 0.7 μM hepcidin for 6 h (*secondary incubation*; Fig. 1*A*). This hepcidin concentration was used initially herein as it has been defined in cell culture studies as the median inhibitory concentration to reduce Fpn1 expression in a seminal publication (5). Subsequently, 0.7 μM was then implemented by numerous groups to assess hepcidin activity (26, 27, 44). Notably, serum-free media were utilized to avoid the binding of hepcidin to bovine serum proteins present in FCS, such as bovine α_2M or bovine albumin.

In the absence of hepcidin, Fpn1 expression was markedly and significantly ($p < 0.001$) enhanced after incubation with FAC relative to CON medium (Fig. 1*A, cf. lanes 2* and *4*). More importantly, the addition of hepcidin not only significantly ($p < 0.001$) decreased Fpn1 expression in CON cells (Fig. 1*A, cf. lanes 2* and *3*), but FAC-induced Fpn1 expression was also markedly and significantly ($p < 0.001$) decreased after incubation with hepcidin relative to FAC-treated cells without hepcidin treatment (Fig. 1*A, cf. lanes 4* and *5*). Collectively, these results confirmed Fpn1 regulation by iron and hepcidin (5, 26, 38, 40).

Hepcidin Complexed with α_2M or α_2M-MA Acts Similarly to Decrease Fpn1 in Vitro—Our previous results demonstrated that hepcidin (0.7 μM) complexed with α_2M (2.8 μM) significantly reduced Fpn1 expression in J774 cells (14). Under physiological conditions, α_2M exists in both native and (to a lesser extent) activated forms and can modulate activity of its bound ligands (9–13). However, we previously only examined the decrease of Fpn1 expression in J774 cells by hepcidin-bound to native α_2M (14). Hence, we investigated the relative efficacy of hepcidin bound to both activated-α_2M and native α_2M at decreasing Fpn1 expression.

Activation of α_2M was achieved by the established procedure of treatment with methylamine (200 mM) (9, 10, 13, 18–20, 28), leading to α_2M-MA. Notably, α_2M-MA closely resembles the structure and function of protease-activated α_2M, and hence, it is widely utilized to mimic the activated state of this protein (19, 20). Activation of α_2M triggers a conformational change resulting in a shift of migration as resolved by native PAGE, with the fast band representing α_2M-MA and the slow band being native α_2M (Fig. 1*B*). The slight overlap in migration of the slow α_2M and fast α_2M-MA bands (Fig. 1*B*) suggests preparations of native α_2M and α_2M-MA contain small proportions of activated and native α_2M, respectively. This observation was not unexpected, as α_2M purified from human plasma contains traces of protease-activated α_2M (45), and methylamine may not activate all α_2M.

In further experiments examining the effects of hepcidin, α_2M·hepcidin, or α_2M-MA·hepcidin on Fpn1 expression, we used cells preincubated (primary incubation) with FAC for 24 h at 37 °C to enhance Fpn1 expression (26) relative to the CON, followed by the 6-h secondary incubation period, again in the absence of serum (Figs. 1–4, 6, and 7). As shown in Fig. 1*A*, unbound hepcidin (0.7 μM) was able to significantly ($p < 0.001$) reduce FAC-induced Fpn1 expression relative to cells treated with FAC without hepcidin (Fig. 1*C, cf. lanes 3* and *4*).

Consistent with our previous study (14), α_2M alone also significantly ($p < 0.05$) reduced Fpn1 levels relative to cells incubated with FAC (Fig. 1*C, cf. lanes 3* and *5*) but to a lesser extent than that elicited by unbound hepcidin (Fig. 1*C, cf. lanes 4* and *5*). This effect could be due to some contaminating endogenous hepcidin bound to purified α_2M, as suggested previously (14). In contrast, no significant ($p > 0.05$) reduction in Fpn1 expression was observed in FAC-treated cells incubated with α_2M-MA relative to FAC-treated cells incubated without hepcidin (Fig. 1*C, cf. lanes 3* and *7*). A potential explanation for this finding is that pre-existing hepcidin bound to native α_2M was released during the methylamine-triggered conformational change (19, 20), which was subsequently removed by Sephadex G-25 filtration column. Both α_2M·hepcidin (*lane 6*) and α_2M-MA·hepcidin (*lane 8*) were able to significantly ($p < 0.001$) reduce Fpn1 expression in J774 cells to a similar extent as hepcidin alone (Fig. 1*C, lane 4*). However, unbound hepcidin is a low molecular weight (~2.8 kDa) peptide (2), which *in vivo* would be readily filtered through the kidney (2, 46, 47). Therefore, hepcidin binding to the high molecular weight α_2M protein (~725 kDa) may prevent its rapid filtration, and this is assessed later below.

Unbound Hepcidin Is Trapped by Size Exclusion Gel Filtration, and α_2M·Hepcidin and α_2M-MA·Hepcidin Complexes Retain Their Ability to Decrease Fpn1 Expression—Although α_2M and α_2M-MA bind hepcidin with appreciable affinity (14), critically it could be suggested that the ability of the α_2M·hepcidin and α_2M-MA·hepcidin complexes to down-regulate Fpn1 expression may be explained by the nonspecific adsorption of hepcidin to the protein and its passive release. To investigate this, α_2M·hepcidin and α_2M-MA·hepcidin complexes were passed through Sephadex G-25 (G-25) columns (retarding molecules ≤5 kDa) immediately prior to incubation with cells. After this procedure, the eluate from the column should be free of unbound hepcidin. Indeed, as shown in Fig. 2, *A* and *B*, although unbound hepcidin significantly ($p < 0.001$) reduced Fpn1 expression relative to cells incubated without hepcidin (Fig. 2, *A* and *B*, *cf. lanes 2* and *3*), treatment of cells with the eluate of Sephadex G-25-filtered hepcidin (*hepcidin(G-25)*) did not significantly ($p > 0.05$) down-regulate Fpn1 (Fig. 2, *A* and *B*, *cf. lanes 2* and *4*). This finding indicates that unbound hepcidin was removed by the column, and thus, the eluate, which was free of hepcidin, had no effect on Fpn1 expression.

In contrast, the eluate derived from passing α_2M·hepcidin (Fig. 2*A*) or α_2M-MA·hepcidin (Fig. 2*B*) complexes (0.7 μM hepcidin complexed to 2.8 μM α_2M or α_2M-MA; 1:4 molar ratio) through the Sephadex G-25 column (*α_2M·hepcidin(G-25)*; Fig. 2*A, lane 7*, or *α_2M-MA·hepcidin(G-25)*; Fig. 2*B, lane 7*)

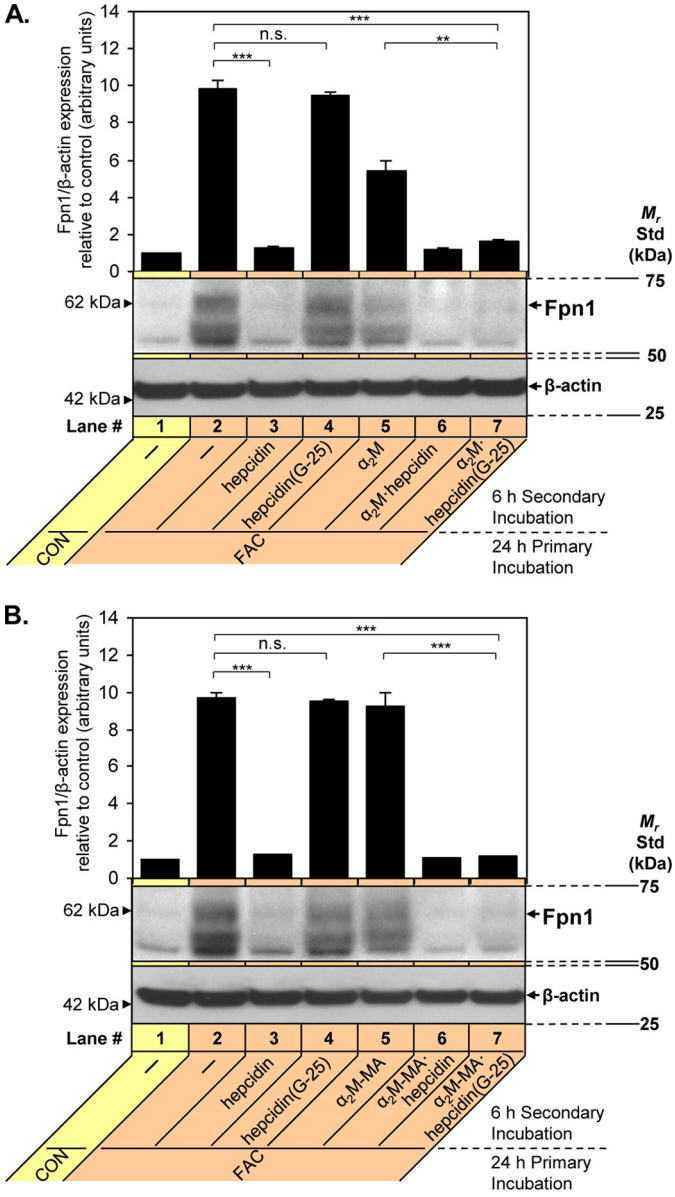

FIGURE 2. **Unbound hepcidin is trapped by Sephadex G-25 size exclusion gel filtration, although α_2M·hepcidin or α_2M-MA·hepcidin complexes retain the ability to decrease Fpn1 expression.** Results for α_2M (*A*) and α_2M-MA (*B*) are shown. Western blot demonstrating the effects of Sephadex G-25 filtration on the ability of hepcidin, α_2M, or α_2M-MA to decrease Fpn1 expression in J774 cells. J774 cells were preincubated with CON media or this medium containing FAC (250 μg/ml) for 24 h at 37 °C (*primary incubation*). Cells were then incubated 6 h at 37 °C (*secondary incubation*) in serum-free media with the following that were filtered through Sephadex G-25, namely hepcidin (hepcidin(G-25); 0.7 μM), α_2M·hepcidin (α_2M·hepcidin(G-25); 2.8 μM α_2M, 0.7 μM hepcidin), α_2M-MA·hepcidin (α_2M-MA·hepcidin(G-25); 2.8 μM α_2M-MA, 0.7 μM hepcidin); and the activity compared with these molecules that were not filtered through Sephadex G-25 at the same concentration. Note: the indicated concentration of hepcidin(G-25) is that prior to being added to the Sephadex column which prevents hepcidin from entering the eluate. The Westerns shown in *A* and *B* are typical blots from three experiments, and the densitometry is mean ± S.D. (three experiments.) *n.s.*, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

markedly and significantly ($p < 0.001$) reduced Fpn1 expression when compared with either FAC alone (Fig. 2, *A* and *B, lane 2*), α_2M (Fig. 2*A, lane 5*), or α_2M-MA (Fig. 2*B, lane 5*). This is because the high molecular weight of α_2M or α_2M-MA with hepcidin-bound passes through the column into the eluate and is not trapped like low molecular weight hepcidin. Notably, the passage of α_2M·hepcidin or α_2M-MA·hepcidin complexes through the Sephadex G-25 column had little effect on their ability to decrease Fpn1 expression (Fig. 2, *A* and *B, cf. lanes 6* and *7*). This is consistent with hepcidin being specifically bound to the protein (14) rather than nonspecifically associated.

Relative Efficacy of Unbound Hepcidin Versus α_2M·Hepcidin and α_2M-MA·Hepcidin at Decreasing Fpn1 Expression—To compare the relative efficacy of unbound hepcidin to α_2M·hepcidin and α_2M-MA·hepcidin at decreasing Fpn1 expression in J774 cells, a concentration curve was assessed. In these studies, FAC-pretreated J774 cells were incubated with increasing concentrations of hepcidin, hepcidin(G-25), α_2M, α_2M·hepcidin (G-25), α_2M-MA, or α_2M-MA·hepcidin (G-25) for 6 h at 37 °C (Fig. 3). The indicated concentration of hepcidin was incubated with and without a 4 M excess of α_2M/α_2M-MA for 1 h at 37 °C immediately prior to treatment of J774 cells. As protein controls, the same concentration of α_2M and α_2M-MA was added to cells without hepcidin at each dose.

As expected from previous studies (5), unbound hepcidin caused a concentration-dependent decrease in Fpn1 expression, with a significant ($p < 0.01$) reduction being observed at hepcidin concentrations ≥0.01 μM (Fig. 3). In contrast, after passage of hepcidin through a Sephadex G-25 column, the hepcidin(G-25) eluate across all concentrations (Fig. 3, *lanes 3–8*) showed no significant reduction in Fpn1 expression relative to FAC alone (*lane 2*), consistent with hepcidin being trapped in the column. Incubation with α_2M alone significantly ($p < 0.05$) decreased Fpn1 from 0.05 μM (Fig. 3, *lanes 5–8*), and α_2M-MA alone at all concentrations (Fig. 3, *lanes 3–8*) did not significantly reduce Fpn1 expression relative to FAC alone (Fig. 3, *lane 2*). Again, these results with α_2M and α_2M-MA could be due to the presence of contaminating endogenous hepcidin presence in α_2M, although this peptide may be released upon activation (19, 20).

In comparison, α_2M·hepcidin and α_2M-MA·hepcidin were able to elute through the Sephadex G-25 column (α_2M·hepcidin (G-25) and α_2M-MA·hepcidin (G-25)) and reduce Fpn1 expression in a concentration-dependent manner (Fig. 3). A significant ($p < 0.05–0.001$) reduction in Fpn1 expression was observed at concentrations of α_2M·hepcidin (G-25) and α_2M-MA·hepcidin (G-25) at ≥0.01 μM. These protein-bound forms of hepcidin were less effective than unbound hepcidin at concentrations of 0.01–0.3 μM (Fig. 3, *lanes 4–7*). However, notably, as the α_2M·hepcidin (G-25) and α_2M-MA·hepcidin (G-25) concentration increased to 0.7 μM (Fig. 3, *lane 8*), there was no significant difference between free hepcidin and that bound to α_2M·hepcidin (G-25) or α_2M-MA·hepcidin (G-25) (Fig. 3).

Collectively, these results show that although unbound hepcidin was trapped within Sephadex G-25, α_2M and α_2M-MA bind hepcidin and allow its elution through the G-25 column. These complexes retain activity and are able to reduce Fpn1 expression in J774 cells, albeit with less efficacy than unbound hepcidin at physiologically relevant hepcidin levels (0.01–0.1 μM (48)).

Eluate from Ultrafiltration of the α_2M-MA·Hepcidin Complex Demonstrates Activity at Decreasing Fpn1 Expression—To assess if the bound hepcidin in Sephadex G-25-filtered

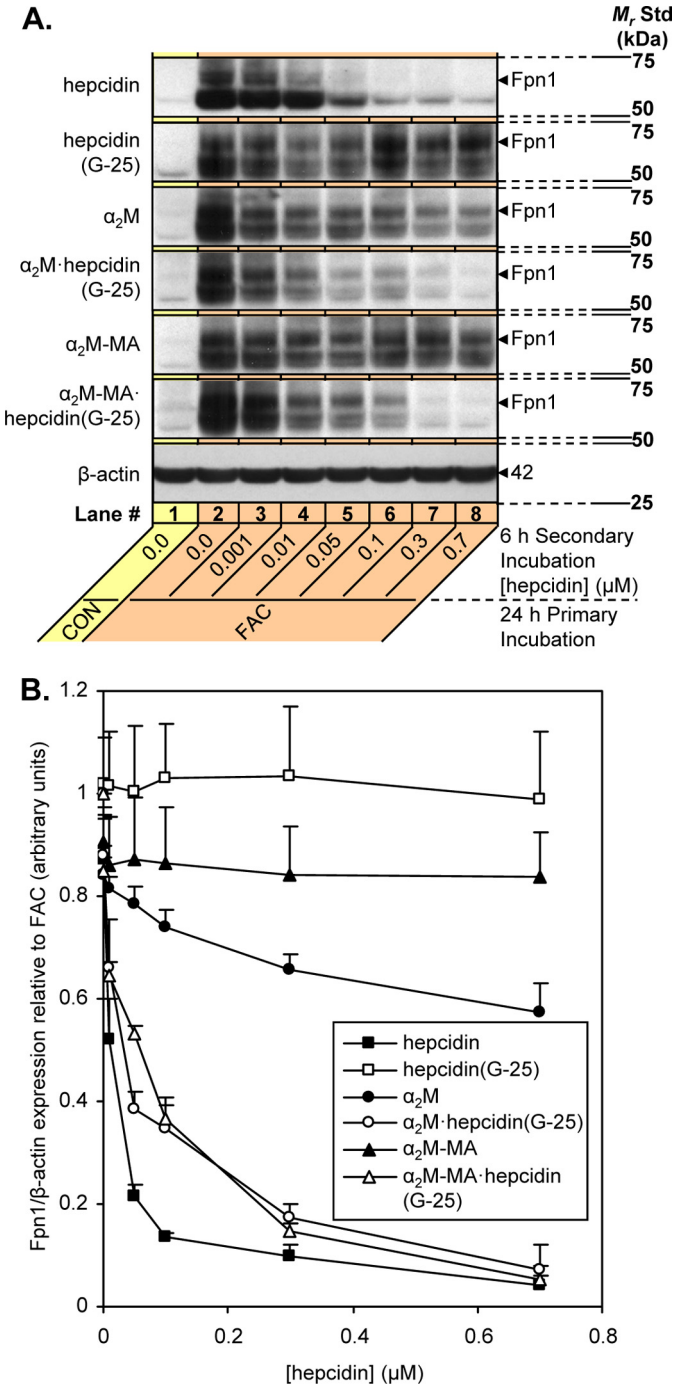

FIGURE 3. **Concentration curve demonstrating Sephadex G-25-filtered α_2M·hepcidin/α_2M-MA·hepcidin complexes dose-dependently elicit Fpn1 reduction in J774 cells.** *A,* Western blot, and *B,* densitometric analysis. J744 cells were incubated as per the procedure in Fig. 2. Note: the indicated concentration of hepcidin(G-25) is that prior to being added to the Sephadex column, which prevents hepcidin from entering the eluate. The Western analyses shown in *A* are typical blots from three experiments, and the densitometric values in *B* are mean ± S.D. (three experiments).

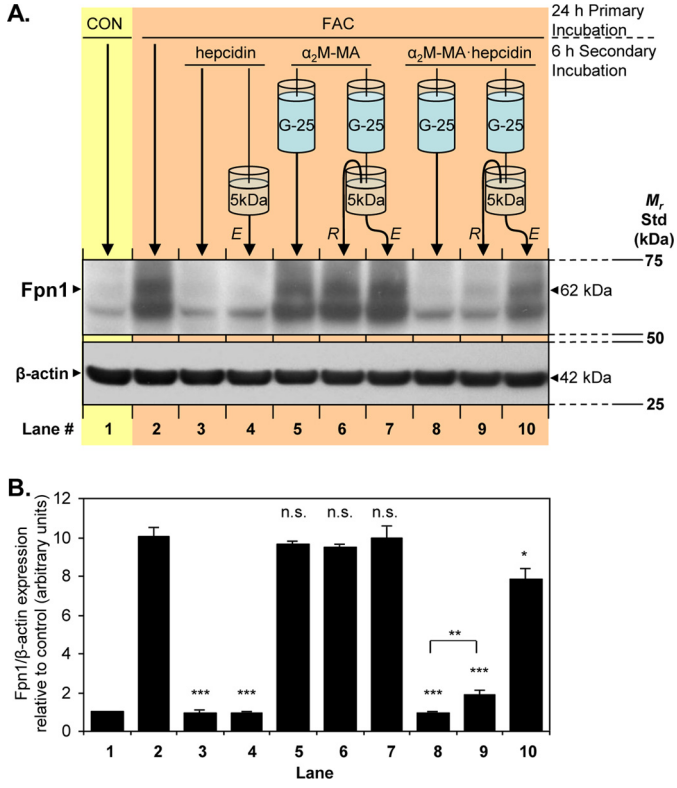

FIGURE 4. **α_2M·hepcidin/α_2M-MA·hepcidin complexes retain activity in decreasing Fpn1 after ultrafiltration (>5 kDa), with some hepcidin being labile.** *A,* experimental procedure and Western blot, and *B,* densitometric analysis. J744 cells were preincubated for 24 h at 37 °C (primary incubation) with control (*CON*) media or this medium containing FAC as per the procedure in Fig. 2, followed by a subsequent 6 h at 37 °C incubation (secondary incubation) with the cell treatments indicated. Western analyses shown in *A* are typical blots from three experiments, and the densitometric values in *B* are mean ± S.D. (three experiments). *n.s.*, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *R*, retained fraction; *E*, eluted fraction.

α_2M·hepcidin or α_2M-MA·hepcidin can dissociate from the complex, we subjected it to a subsequent 5-kDa ultrafiltration step. As depicted in Fig. 4*A*, J774 cells preincubated with FAC were treated with either hepcidin (0.7 μM), α_2M-MA (2.8 μM), or α_2M-MA·hepcidin (2.8 μM α_2M-MA: 0.7 μM hepcidin) subjected to the Sephadex G-25 column, 5-kDa ultrafiltration, or both. As expected, the low molecular weight unbound hepcidin (~2.8 kDa; Fig. 4, *lane 4*) eluted through the 5-kDa ultrafilter and significantly ($p < 0.001$) reduced Fpn1 expression to a comparable extent to hepcidin without ultrafiltration (Fig. 4, *cf. lanes 3* and *4*).

The Sephadex G-25-filtered high molecular weight α_2M-MA alone (without hepcidin bound) that was retained through the subsequent 5-kDa ultrafiltration step showed no significant effect on Fpn1 expression compared with FAC-treated control cells (Fig. 4, *cf. lanes 2* and *6*). Similarly, the eluted fraction from these experiments showed no activity (Fig. 4, *cf. lanes 2* and *7*), as the α_2M-MA used in this treatment condition is devoid of hepcidin.

The fraction of Sephadex G-25-filtered high molecular weight α_2M-MA·hepcidin complex retained by the 5-kDa ultrafilter elicited a significant ($p < 0.001$) reduction in Fpn1 expression relative to FAC-treated control cells (Fig. 4, *cf. lanes 2* and *9*). However, there was a slight but significant ($p < 0.01$) loss of activity when comparing this latter treatment to when the α_2M-MA·hepcidin complex was just Sephadex G-25-filtered (Fig. 4, *cf. lanes 8* and *9*). Furthermore, the eluted fraction from the ultrafiltered Sephadex G-25 α_2M-MA·hepcidin complex also resulted in a slight but significant ($p < 0.05$) decrease in Fpn1 expression relative to FAC-treated control (Fig. 4, *cf.*

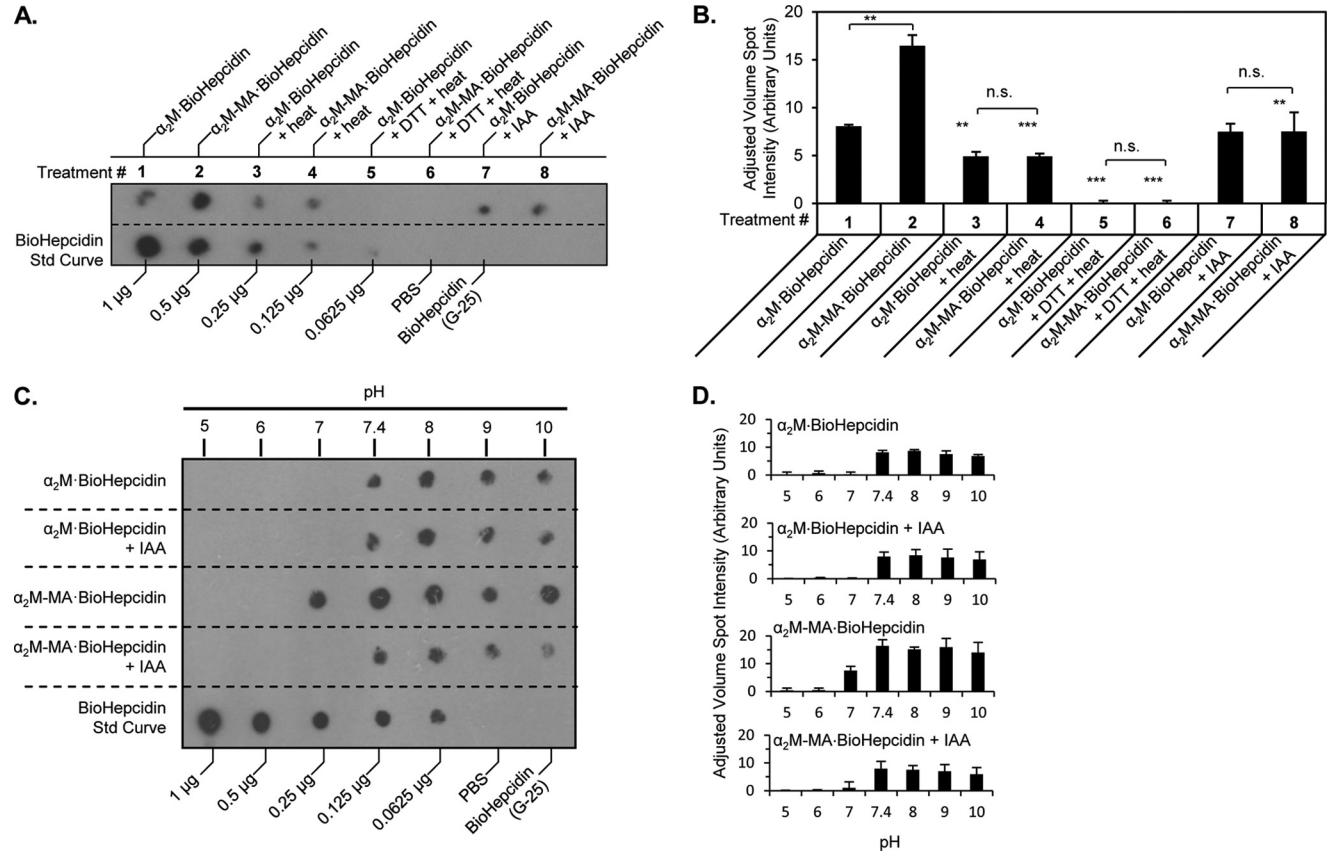

FIGURE 5. **Hepcidin complexation to α_2M or α_2M-MA can be altered by IAA, DTT, heat, and pH.** *A* and *B,* for IAA experiments, α_2M/α_2M-MA were treated with or without 2 mM IAA (22 °C for 1 h), with unreacted IAA subsequently removed by passage through a Sephadex G-25 column. Then, BioHepcidin was complexed to α_2M/α_2M-MA followed by passage through a Sephadex G-25 to remove unbound BioHepcidin. For heat and DTT experiments, the α_2M·BioHepcidin and α_2M-MA·BioHepcidin complexes were treated with or without heat (100 °C for 10 min) in the absence or presence of 2 mM DTT. After a further round of Sephadex G-25 column chromatography to remove released BioHepcidin and DTT, protein complexes were spotted onto nitrocellulose membrane, where biotin was detected via chemiluminescence. *C* and *D,* α_2M/α_2M-MA (or these proteins treated with IAA as above) were dissolved and incubated at pH 5, 6, 7, 7.4, 8, 9, and 10 (using the buffers described under "Experimental Procedures"). BioHepcidin was then added to the α_2M/α_2M-MA protein as described above. The proteins were then applied to the membrane and detected by chemiluminescence. The dot blot analyse shown in *A* and *C* are typical from three experiments, and the densitometric values in *B* and *D* are mean ± S.D. (three experiments). *n.s.*, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

lanes 2 and *10*). These results suggest that due to the equilibrium between protein-bound and unbound hepcidin (14), there was some dissociation of hepcidin from the α_2M-MA·hepcidin complex that was subsequently eluted through the 5-kDa ultrafilter, resulting in a slight loss of activity in the retained fraction (Fig. 4, *lane 9*). Such dissociation of hepcidin from the complex demonstrates its labile binding and could explain urinary hepcidin excretion (2). Similar results were also obtained using α_2M·hepcidin (data not shown). Hence, potentially, the presence of Fpn1 on cells may drive the equilibrium toward release of hepcidin from its complexation with α_2M or α_2M-MA.

Hepcidin Binds to α_2M/α_2M-MA via a Mechanism Directly or Indirectly Involving Thiols—Collectively, the results above demonstrate that hepcidin binds to α_2M and α_2M-MA and is active in terms of down-regulating Fpn1. To determine the specific nature of the interaction, BioHepcidin was incubated at a 2:1 molar ratio of BioHepcidin:α_2M and 4:1 BioHepcidin:α_2M-MA. To assess the mechanism of hepcidin binding to α_2M/α_2M-MA, the following studies were performed via a blotting method (see under "Experimental Procedures") using three treatment conditions as follows: 1) heat; 2) dithiothreitol (DTT) and heat; and 3) IAA, as performed previously for other ligands (17, 31).

As we demonstrated using other methods (14), there was 2-fold more BioHepcidin binding to α_2M-MA than α_2M (Fig. 5, *A* and *B*, *cf. treatments 1* and *2*). Heating (100 °C/10 min), significantly ($p < 0.001$) reduced binding of BioHepcidin to α_2M-MA (Fig. 5, *A* and *B*, *cf. treatments 2* and *4*) suggesting the conformational change upon denaturation led to BioHepcidin release. A slight but significant ($p < 0.01$) loss of BioHepcidin was also observed in heat-treated α_2M·hepcidin complexes *versus* the nontreated control (Fig. 5, *A* and *B*, *cf. treatments 1* and *3*).

Studies then examined the effect of the disulfide reductant, DTT, on hepcidin binding, where α_2M·BioHepcidin or α_2M-MA·BioHepcidin complexes were incubated with 2 mM DTT (100 °C/10 min) (17). Upon DTT + heat treatment, BioHepcidin was almost totally displaced from α_2M and α_2M-MA (Fig. 5, *A* and *B*, *treatments 5* and *6*), suggesting thiol covalent binding in α_2M and α_2M-MA and/or disulfide-dependent tertiary and quaternary structure is vital for hepcidin binding.

To assess if hepcidin binds to α_2M/α_2M-MA via thiols, IAA was used, which blocks thiol binding (Fig. 5, *A* and *B*, *treatments 7* and *8*) (31). In these experiments, α_2M and α_2M-MA were preincubated with 2 mM IAA at 20 °C for 1 h (31), followed by G-25 chromatography to remove unreacted IAA. These proteins were then incubated with BioHepcidin for 1 h at 37 °C (Fig. 5, *A* and *B*). When thiol groups were blocked by IAA, there was a marked and significant ($p < 0.01$) decrease in BioHepcidin binding to α_2M-MA relative to α_2M-MA not treated with IAA (Fig. 5, *A* and *B*, *cf. treatments 2* and *8*), suggesting the importance of thiols exposed after activation (18). In contrast, there was no significant effect of IAA on α_2M (Fig. 5, *A* and *B*, *cf. treatments 1* and *7*), which is consistent with the lack of free thiol groups in this protein (49). Notably, the decrease in binding mediated by IAA in α_2M-MA relative to α_2M led to similar levels of BioHepcidin retention (Fig. 5, *A* and *B*, *cf. treatments 7* and *8*). The later IAA data suggest that the binding of BioHepcidin to α_2M involves noncovalent (non-thiol) interactions, although in α_2M-MA there is also involvement of thiol-dependent interactions. These studies agree with the classical theory indicating that thiol groups become available when α_2M is activated (18).

The lability of hepcidin binding to α_2M/α_2M-MA was assessed as a function of pH (pH 5 to 10). Maximal BioHepcidin binding to α_2M and α_2M-MA was generally observed between a pH of 7.4 and 8 (Fig. 5, *C* and *D*). At higher pH values, slightly decreased BioHepcidin binding was observed, potentially due to denaturation. Interestingly, even slight acidification from pH 7.4 to 7 resulted in a marked and significant ($p < 0.01$) decrease in BioHepcidin binding to α_2M. In contrast, for α_2M-MA, the BioHepcidin binding at pH 7 was significantly ($p < 0.001$) greater than that found for α_2M. This resistance of α_2M-MA in releasing the peptide suggested a different mode of binding relative to α_2M. However, for both α_2M and α_2M-MA, at pH 6 and below, there was no binding of BioHepcidin to the protein (Fig. 5, *C* and *D*). These data demonstrate that BioHepcidin binding to α_2M or α_2M-MA is highly pH-dependent and indicates the interaction is relatively labile, supporting the results in Fig. 4.

In an attempt to understand the mechanism responsible for the relative resistance of α_2M-MA to release BioHepcidin upon acidification, α_2M and α_2M-MA were treated with IAA to assess the potential role of thiol interactions observed in Fig. 5*A*. Pretreatment of α_2M with IAA had no effect on BioHepcidin-binding (as illustrated in Fig. 5, *A* and *B*) nor did it significantly affect pH-dependent binding relative to nontreated α_2M (Fig. 5, *C* and *D*). This is because native α_2M does not possess free thiols (49). As shown in Fig. 5, *A* and *B*, preincubation of α_2M-MA with IAA decreased BioHepcidin binding to approximately half. Moreover, it led to a similar pH-dependent BioHepcidin-binding profile as that found with native α_2M pretreated with IAA. This observation suggested that blocking thiols in α_2M-MA not only decreased BioHepcidin binding but also prevented the greater resistance of α_2M-MA in releasing hepcidin. Hence, thiol-dependent interactions in α_2M-MA may be responsible for resistance to BioHepcidin release upon acidification (Fig. 5, *C* and *D*).

Collectively, the data above suggest a greater proportion of BioHepcidin is bound in α_2M-MA relative to α_2M by heat-sensitive and thiol-dependent interactions and that the tertiary and/or quaternary structure of α_2M/α_2M-MA is vital for BioHepcidin binding. Thiols may be involved in direct hepcidin binding, or thiol modification could lead to changes in α_2M-MA conformation that indirectly affects hepcidin binding. Considering these hypotheses, from the evidence of labile hepcidin binding (Fig. 4) and the marked dependence of hepcidin binding to α_2M/α_2M-MA on pH (Fig. 5, *C* and *D*), we propose that hepcidin binding does not involve direct covalent interaction with thiols.

Only ^{125}I-α_2M-MA Is Markedly Internalized by Lrp1$^{+/+}$ Cells and Hepcidin Had No Effect on This Mechanism—As demonstrated in Figs. 1*C* and 2–4, both α_2M·hepcidin and α_2M-MA·hepcidin decrease Fpn1 expression. However, the role α_2M/α_2M-MA plays in facilitating the hepcidin-Fpn1 interaction remains unknown and was investigated below.

Previous studies investigating hormone binding by α_2M or α_2M-MA demonstrated the involvement of the α_2M receptor (Lrp1) in cellular uptake of hormone·α_2M-MA complexes (10, 50). Lrp1 is the major receptor responsible for clearance of activated-α_2M from the circulation (18). As Lrp1 is unable to bind native α_2M (18, 51), the mechanism responsible for the equipotent decrease in Fpn1 expression by α_2M·hepcidin and also α_2M-MA·hepcidin complexes (Figs. 1*C*, 2, and 3) warranted investigation. Thus, to assess the role of Lrp1 in facilitating the hepcidin-mediated reduction in Fpn1 expression, MEFs from wild-type (*Lrp1*$^{+/+}$) and knock-out (*Lrp1*$^{-/-}$) mice were utilized (Fig. 6*A*). Initial studies examined uptake of ^{125}I-labeled α_2M compared with ^{125}I-α_2M-MA in these cells in the absence or presence of nonradiolabeled hepcidin (Fig. 6*B*).

The internalization of ^{125}I-α_2M-MA was markedly and significantly ($p < 0.001$) greater than ^{125}I-α_2M in *Lrp1*$^{+/+}$ cells (Fig. 6*B*, *cf. treatments 1* and *3*). Notably, hepcidin binding did not significantly ($p > 0.05$) affect ^{125}I-α_2M or ^{125}I-α_2M-MA internalization by Lrp1 (Fig. 6*B*, *cf. treatments 1* and *2* with *3* and *4*). As discussed above, Lrp1 only binds and internalizes activated-α_2M (18, 51). Thus, the small amount of ^{125}I-α_2M internalization observed in Fig. 6*B* (*treatments 1* and *2*) could be due to the minor amounts of activated α_2M in commercial α_2M preparations, as demonstrated in Fig. 1*B*. In contrast to *Lrp1*$^{+/+}$ cells, *Lrp1*$^{-/-}$ cells demonstrated no significant internalization of ^{125}I-α_2M-MA or ^{125}I-α_2M (Fig. 6*B*, *treatments 5–8*).

Examining *Lrp1*$^{+/+}$ cells, the membrane-associated ^{125}I-α_2M-MA fraction constituted only ~12% of the total uptake of the molecule (Fig. 6*B*, *treatments 3* and *4*). However, the membrane association of ^{125}I-α_2M-MA by the *Lrp1*$^{+/+}$ cells was 3-fold greater than that for ^{125}I-α_2M (Fig. 6*B*, *cf. treatments 3* and *4* with *1* and *2*). This observation suggested specific receptor binding on the plasma membrane. There was very little membrane binding of either ^{125}I-α_2M-MA or ^{125}I-α_2M to *Lrp1*$^{-/-}$ cells (Fig. 6*B*, *treatments 5–8*), and quantitatively, it was similar to ^{125}I-α_2M membrane binding in *Lrp1*$^{+/+}$ cells (Fig. 6*B*, *treatments 1* and *2*), suggesting that it represented nonspecific binding to the cell membrane. In fact, there was no significant difference in the membrane binding of ^{125}I-α_2M-MA compared with ^{125}I-α_2M to *Lrp1*$^{-/-}$ cells (Fig. 6*B*, *cf. treatments 7* and *8* with *5* and *6*). This observation indicates that another putative receptor, namely the activated α_2M-signaling

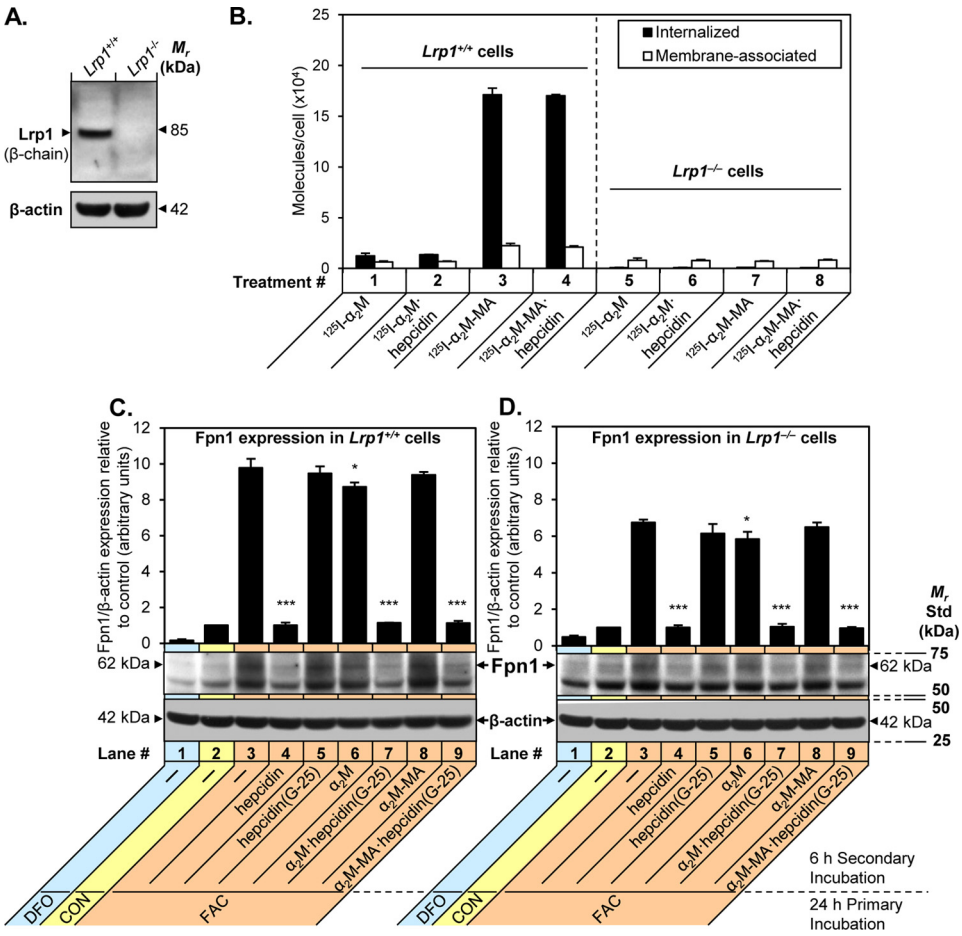

FIGURE 6. **Reduction of Fpn1 expression by α_2M·hepcidin/α_2M-MA·hepcidin complexes was an Lrp1-independent process as shown using *Lrp1*$^{+/+}$/*Lrp1*$^{-/-}$ MEFs.** *A,* Western blot demonstrating Lrp1 expression is only present in *Lrp1*$^{+/+}$ but not in *Lrp1*$^{-/-}$ MEFs. *B, Lrp1*$^{+/+}$/*Lrp1*$^{-/-}$ MEFs were incubated with ^{125}I-α_2M (2.8 μM), ^{125}I-α_2M·hepcidin (2.8 μM α_2M, 0.7 μM hepcidin), ^{125}I-α_2M-MA (2.8 μM), or ^{125}I-α_2M-MA·hepcidin (2.8 μM α_2M-MA, 0.7 μM hepcidin) for 1 h at 37 °C. The cells were then washed four times with ice-cold PBS; membrane and internalized uptake was assayed using Pronase. *C,* Western blot and densitometric analysis of Fpn1 expression in *Lrp1*$^{+/+}$ (*C*) and *Lrp1*$^{-/-}$ (*D*) cells. Cells were preincubated for 24 h at 37 °C (*primary incubation*) as described in Fig. 1 with either control (*CON*) medium, DFO (100 μM), or FAC (250 μg/ml). The media were then removed and replaced with media containing the corresponding treatments (CON, DFO, or FAC) in the absence or presence of hepcidin (0.7 μM), hepcidin(G-25) (0.7 μM), α_2M (2.8 μM), α_2M·hepcidin(G-25) (2.8 μM α_2M, 0.7 μM hepcidin), α_2M-MA (2.8 μM), or α_2M-MA·hepcidin(G-25) (2.8 μM α_2M-MA, 0.7 μM hepcidin) for a further 6 h at 37 °C (*secondary incubation*). The Western analyses in *A, C,* and *D* are typical from three experiments, and results in *B* are mean ± S.D. (three experiments). The densitometric values in *C* and *D* are mean ± S.D. (three experiments). *, $p < 0.05$; ***, $p < 0.001$.

receptor, GRP78 (52), has little or no role in the binding of the activated form of α_2M to MEFs. Furthermore, in no case in *Lrp1*$^{+/+}$ or *Lrp1*$^{-/-}$ cells did the binding of hepcidin have any effect on membrane association or internalization of ^{125}I-α_2M or ^{125}I-α_2M-MA (Fig. 6*B*). In summary, only ^{125}I-α_2M-MA was markedly internalized by *Lrp1*$^{+/+}$ cells, and hepcidin had no effect on this process.

α_2M·Hepcidin/α_2M-MA·Hepcidin Induces a Decrease in Fpn1 by a Mechanism Independent of the α_2M Receptor, Lrp1—We then examined the effect of hepcidin and its α_2M or α_2M-MA complexes on Fpn1 expression in *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ cells (Fig. 6, *C* and *D*). Again, cells were preincubated for 24 h at 37 °C with either CON medium or this medium containing DFO (100 μM) or FAC (250 μg/ml). The medium was then removed and replaced with serum-free media containing the corresponding treatments (CON medium or this medium containing DFO or FAC) in the absence or presence of hepcidin (0.7 μM), hepcidin(G-25) (0.7 μM), α_2M (2.8 μM), α_2M-MA (2.8 μM), α_2M·hepcidin (G-25) (2.8 μM α_2M, 0.7 μM hepcidin), or α_2M-MA·hepcidin (G-25) (2.8 μM α_2M-MA, 0.7 μM hepcidin) for a further 6 h at 37 °C.

In *Lrp1*$^{+/+}$ cells (Fig. 6*C*), the Fpn1 expression profile following hepcidin treatments was similar to that in J774 cells (Fig. 1*C*). Importantly, using *Lrp1*$^{-/-}$ cells (Fig. 6*D*), Fpn1 expression also responded to hepcidin treatments in a similar manner to *Lrp1*$^{+/+}$ cells (Fig. 6*C*), albeit the FAC-induced expression of Fpn1 was not as pronounced as that observed in *Lrp1*$^{+/+}$ cells. These results indicate that although the α_2M·hepcidin and α_2M-MA·hepcidin complexes decrease Fpn1 expression in MEF cells, the α_2M receptor, Lrp1, is not essential for hepcidin's ability to reduce Fpn1 expression. Moreover, there was no evidence of any other receptor that played a significant role in uptake of α_2M·hepcidin or α_2M-MA·hepcidin in *Lrp1*$^{-/-}$ cells (Fig. 6*B*).

Lrp1 Down-regulation Using Pretreatment of J774 Cells with α_2M-MA Does Not Affect the Decrease in Fpn1 Mediated by α_2M·Hepcidin or α_2M-MA·Hepcidin—As a second assessment of the role of Lrp1 in the hepcidin-mediated down-regulation of

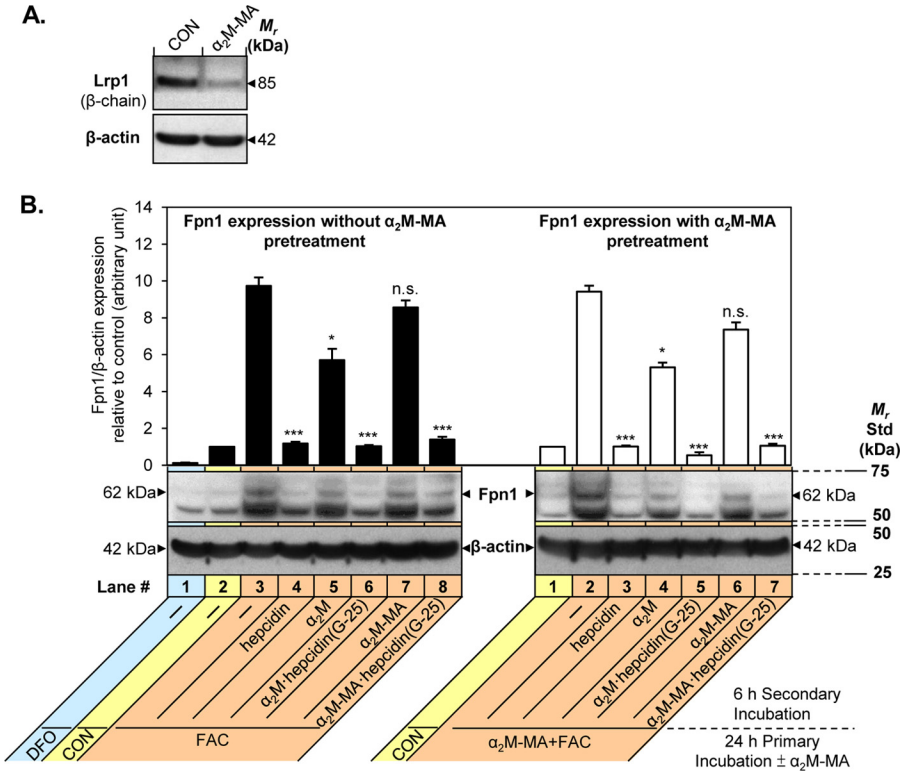

FIGURE 7. **Preincubation of J774 with α_2M-MA down-regulates Lrp1 expression but does not affect Fpn1 down-regulation by either hepcidin or hepcidin complexed to α_2M and α_2M-MA.** *A*, saturating endogenous Lrp1 with a high pretreatment dose of α_2M-MA (2.8 μM) for 24 h results in processing and removal of Lrp1/α_2M-MA from the cell surface. After this pretreatment with medium alone (control) or medium containing α_2M-MA, the medium was replaced with control media for a further 6 h, and Lrp1 expression was assessed by Western blot. *B*, Fpn1 expression in J774 cells after a 24-h preincubation with or without α_2M-MA (2.8 μM) in the presence or absence of either control (*CON*), DFO (100 μM), or FAC (250 μg/ml). The incubation media were then removed, and the cells then subjected to a 6 h at 37 °C incubation in serum-free media (CON or FAC, respectively) in the absence or presence of 0.7 μM hepcidin, 0.7 μM hepcidin(G-25) , 2.8 μM α_2M/α_2M-MA or α_2M·hepcidin(G-25)/α_2M-MA·hepcidin(G-25) , as indicated. The Western analyses in *A* and *B* are typical from three experiments, and the densitometric values in *A* and *B* are mean ± S.D. (three experiments). *, $p < 0.05$; ***, $p < 0.001$.

Fpn1 in another cell type, we markedly decreased Lrp1 expression in J774 cells via a 24-h preincubation with α_2M-MA (Fig. 7*A*). Saturating endogenous Lrp1 with a high pretreatment dose of α_2M-MA (2.8 μM) for 24 h results in processing and removal of Lrp1/α_2M-MA from the cell surface via receptor-mediated endocytosis (51, 53). This preincubation effectively down-regulates cell surface Lrp1 for subsequent experiments. After pretreatment with medium alone (Control) or medium containing α_2M-MA, this medium was replaced with control media for a further 6 h. As a result of α_2M-MA pretreatment, Lrp1 expression was markedly and significantly ($p < 0.001$) reduced to ~20% of the untreated control (Fig. 7*A*). As evident in Fig. 7*B*, Lrp1 down-regulation after α_2M-MA pretreatment did not significantly affect hepcidin-induced Fpn1 degradation by unbound hepcidin or that bound to α_2M or α_2M-MA relative to control cells. Hence, together with the studies using *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ cells (Fig. 6), these results demonstrate Fpn1 degradation by α_2M·hepcidin and α_2M-MA·hepcidin complexes is an Lrp1-independent process.

Plasma Clearance Studies Examining Circulatory Clearance of α_2M/α_2M-MA with and without Bound Hepcidin—To examine α_2M·hepcidin/α_2M-MA·hepcidin interactions *in vivo*, plasma clearance studies were conducted in mice intravenously injected with ^{125}I-α_2M or ^{125}I-α_2M-MA (0.36 nmol each) with or without bound hepcidin (0.09 nmol; Fig. 8*A*). As reported previously (8, 36, 37, 50), ^{125}I-α_2M-MA was rapidly cleared from the circulation ($t_{1/2}$ <1 min), whereas the clearance of ^{125}I-α_2M was markedly slower with 79.9% of initial radioactivity remaining after 60 min (Fig. 8*A*). In contrast, injection of ^{125}I was almost instantaneously cleared from the circulation with ~0.05% radioactivity remaining after 1 min (Fig. 8*A*). Consistent with our *in vitro* results (Fig. 6*B*), hepcidin binding to ^{125}I-α_2M-MA and ^{125}I-α_2M did not significantly alter their clearance from the circulation (Fig. 8*A*).

Hypoferremic Effect of Hepcidin Is Accentuated by Complexation to Native α_2M and to a Greater Extent α_2M-MA—To assess the effectiveness of unbound hepcidin relative to that bound to α_2M and α_2M-MA *in vivo*, unbound hepcidin (0.9 nmol) or the same amount complexed to a 4-fold excess of α_2M and α_2M-MA (3.6 nmol) was intravenously injected into mice. Blood was then collected 2 h post-injection and analyzed for serum iron. As shown previously (47), hepcidin resulted in a significant ($p < 0.001$) reduction in serum iron compared with the vehicle-treated control (Fig. 8*B*). An equivalent amount of hepcidin (0.9 nmol) bound to α_2M or α_2M-MA resulted in a further significant ($p < 0.01$–0.001) decrease in serum iron compared with unbound hepcidin alone. In contrast, equivalent amounts of α_2M or α_2M-MA alone (3.6 nmol) did not elicit any significant ($p > 0.05$) decrease in serum iron (Fig. 8*B*). Notably, the α_2M-MA·hepcidin complex was significantly ($p < 0.05$) more effective in reducing serum iron than α_2M·hepcidin. This *in vivo* result complements our *in vitro* data and demon-

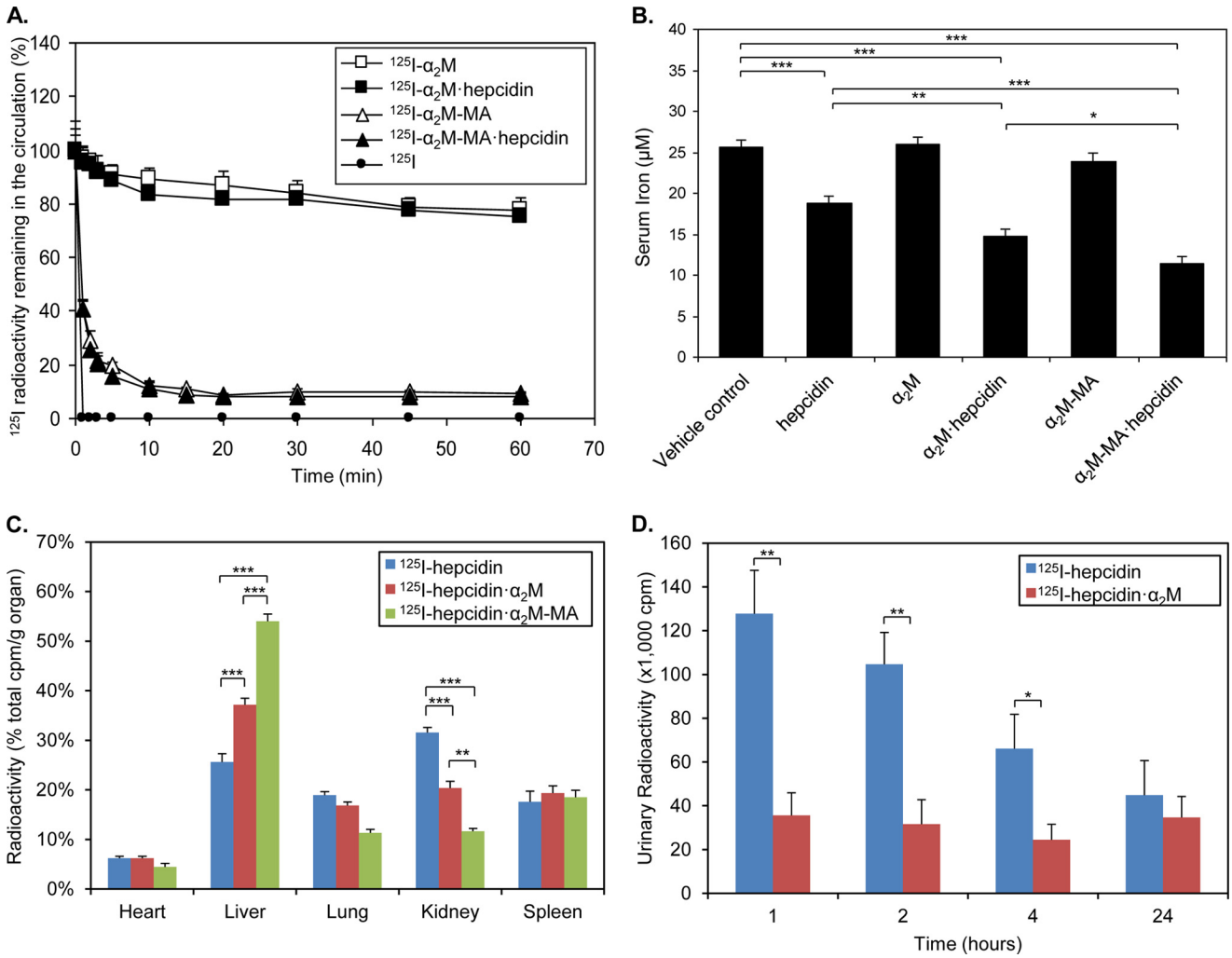

FIGURE 8. **Hepcidin binding does not affect the clearance of α_2M or α_2M-MA from the circulation, although complexation of hepcidin with α_2M and α_2M-MA enhances the hypoferremic effect of hepcidin and retards the urinary excretion of the peptide.** *A,* mice were intravenously injected with ^{125}I-α_2M (0.36 nmol), ^{125}I-α_2M-MA (0.36 nmol), ^{125}I-α_2M·hepcidin complex (0.36 nmol α_2M, 0.09 nmol of hepcidin), ^{125}I-α_2M-MA·hepcidin complex (0.36 nmol α_2M, 0.09 nmol of hepcidin), or ^{125}I (2.5 μCi). Mice were bled by tail snip at the times shown, and ^{125}I was measured. Radioactivity remaining in the circulation was calculated as described under "Experimental Procedures." Results shown are mean ± S.E. (eight mice/group). *B,* mice received a single 0.9 nmol dose of hepcidin, α_2M·hepcidin/α_2M-MA·hepcidin complexes (3.6 nmol of α_2M/α_2M-MA, 0.9 nmol of hepcidin) or α_2M/α_2M-MA (3.6 nmol) by intravenous injection. Serum iron concentrations were determined 2 h after the injection. *C* and *D,* mice were intravenously injected with ^{125}I (2.5 μCi), ^{125}I-hepcidin, ^{125}I-hepcidin-α_2M, or ^{125}I-hepcidin-α_2M-MA (proteins, 0.36 nmol; ^{125}I-hepcidin, 0.09 nmol). The ^{125}I radioactivity in the excreted urine was assessed at 1, 2, 4, and 24 h after injection (see "Experimental Procedures" for details). At the terminating time point (*i.e.* 24 h after injection), mice were euthanized, and major organs were collected and washed, and ^{125}I was measured. Results in *A* and *B* are mean ± S.E. (14 mice/group), and those in *C* and *D* are mean ± S.E. (7–8 mice/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

strates hepcidin complexed to α_2M or α_2M-MA remains biologically active. This is probably due to the high molecular weight of the complex (~725 kDa), which is retained in the body, relative to low molecular weight unbound hepcidin (~2.8 kDa), which is efficiently excreted by the kidney.

Notably, the reduced hypoferremic response of free hepcidin compared with preformed α_2M·hepcidin/α_2M-MA·hepcidin complexes (Fig. 8*B*) may reflect lower affinity of human hepcidin to endogenous murine α_2M orthologs, leading to reduced hepcidin activity. Hence, the preformed complex is more effective than free hepcidin.

Organ Uptake of Unbound ^{125}I-Hepcidin and That Bound to α_2M or α_2M-MA—Studies also directly examined the uptake of unbound ^{125}I-hepcidin relative to ^{125}I-hepcidin precomplexed to α_2M or α_2M-MA by major organs in mice 24 h after an intravenous injection (Fig. 8*C*). The uptake of protein-bound or -unbound ^{125}I-hepcidin was predominantly observed in the liver, which is in good agreement with its major role in iron metabolism (54). Notably, the uptake in the liver of ^{125}I-hepcidin complexed to α_2M-MA was significantly ($p < 0.001$) greater than that found for either ^{125}I-hepcidin or that complexed to α_2M. This finding can be explained by the interaction of α_2M-MA with its receptor, Lrp1 (18), which is abundant in the liver (55). Furthermore, the uptake of ^{125}I-hepcidin bound to α_2M in the liver was also significantly ($p < 0.001$) greater than that of ^{125}I-hepcidin alone. In contrast to the other organs, in the kidney ^{125}I-hepcidin uptake was significantly ($p < 0.001$) greater than that of ^{125}I-hepcidin complexed to α_2M or α_2M-MA (Fig. 8*C*). Importantly, this was also reflected in the urine, with ^{125}I-hepcidin alone demonstrating significantly ($p < 0.05$–0.01) greater initial urinary excretion at the 1-, 2-, and 4-h time point relative to ^{125}I-hepcidin bound to α_2M,

which showed low and constant urinary excretion from 1 to 24 h (Fig. 8*D*). This finding indicates a greater excretion of ^{125}I-hepcidin relative to that complexed to α_2M. The excretion of ^{125}I after injection of the α_2M-MA·hepcidin complex is not shown due to its rapid uptake and metabolism by the liver (Fig. 8, *A* and *C*).

DISCUSSION

For the first time, we have assessed the activity of hepcidin bound to both activated α_2M and native α_2M and its effect on Fpn1 expression. This was important considering that activated α_2M binds to the α_2M receptor Lrp1 (18), which may facilitate hepcidin targeting. Indeed, we aimed to ascertain the mechanisms of how α_2M·hepcidin/α_2M-MA·hepcidin complexes regulate iron metabolism.

Incubation of J774 cells with unbound hepcidin confirmed it markedly decreased Fpn1 expression (Fig. 1*A*), as reported (5, 26, 27). However, because of the low molecular weight (~2.8 kDa) and cationic nature of unbound hepcidin (2), it is readily excreted by the kidney (46, 47). Thus, the ability of hepcidin to bind to the high molecular weight serum protein, α_2M (~725 kDa) (14), would retard hepcidin clearance.

Complexation of Hepcidin to α_2M and α_2M-MA Down-regulates Fpn1 at Physiologically Relevant Hepcidin Concentrations—Considering this latter hypothesis, the results herein demonstrated that hepcidin complexed to native or activated α_2M significantly decreased Fpn1 expression (Figs. 1*C*, 2–4, 6 (*C* and *D*), and 7*B*), and this was dependent on hepcidin concentration (Fig. 3). Furthermore, Sephadex G-25 chromatography and 5-kDa ultrafiltration experiments demonstrated that although unbound hepcidin was removed by the former method, hepcidin complexed to α_2M or α_2M-MA remained bound but was shown to demonstrate some lability after ultrafiltration (Fig. 4). Such binding of hepcidin to α_2M and α_2M-MA would allow efficient transport and retard rapid clearance of hepcidin by the kidney but still enable down-regulation of Fpn1. Together with our previous investigation (14), these results demonstrated that 1) α_2M and α_2M-MA bind hepcidin and 2) both these forms retain activity after Sephadex G-25 filtration and/or 5-kDa ultrafiltration, decreasing Fpn1 expression. Considering the physiological levels of hepcidin in the blood (0.01–0.1 μM (48)), it is notable that when hepcidin is complexed to α_2M and α_2M-MA at concentrations of 0.01–0.1 μM, it was markedly effective at reducing Fpn1 expression, indicating the effect observed is physiologically relevant.

In this study, we identified that a greater proportion of hepcidin is bound in α_2M-MA relative to α_2M by heat-sensitive and thiol-dependent interactions. Similar results have been observed for α_2M-MA binding to defensin, HNP1, which is a cysteine-rich, anti-microbial peptide with structural similarities to hepcidin (17, 56). Notably, in both hepcidin and HNP1, all cysteine residues are engaged in intra-molecular disulfide bonds (17, 57). The mechanism of HNP1·α_2M-MA binding suggested by Panyutich and Ganz (17) may involve thiol-disulfide interchange between α_2M-MA and HNP1. Hence, potentially, a similar mechanism may occur between hepcidin and the internal free thiol groups in α_2M-MA after activation (18). However, considering that hepcidin binding to α_2M and

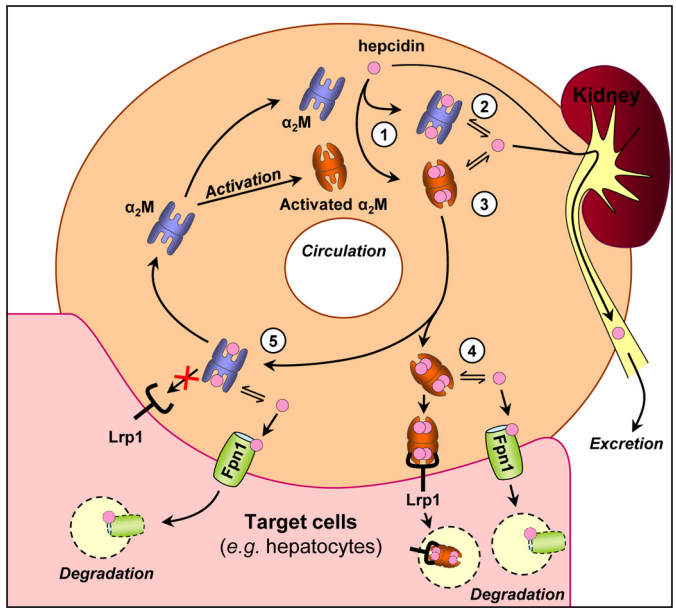

FIGURE 9. **Schematic model illustrates that α_2M in its native and activated forms acts as a hepcidin-carrier protein in the circulation.** *1)* Hepcidin in the circulation binds to native α_2M and the much smaller proportion of activated α_2M that is present under physiological conditions (14–16). *2)* Free hepcidin in equilibrium with that bound to α_2M can be excreted by the kidney due to its low molecular weight (~2.8 kDa). *3)* Binding of hepcidin to high molecular weight (~725 kDa) α_2M or activated α_2M retards kidney filtration. *4)* Hepcidin bound to activated α_2M binds to the α_2M receptor (Lrp1) on target cells potentially facilitating its exposure to Fpn1, and Lrp1 can be internalized. *5)* In contrast to activated α_2M, native α_2M is not bound by Lrp1 (18) extending the half-life of bound hepcidin and enhancing exposure to Fpn1. Considering our studies demonstrating hepcidin release from α_2M-MA in ultrafiltration experiments (Fig. 4, *lane 10*) and that the α_2M receptor, Lrp1, is not involved in reducing Fpn1 expression (Figs. 6 and 7), we propose a labile interaction that facilitates transport of hepcidin by α_2M and α_2M-MA and its release to Fpn1.

α_2M-MA is somewhat labile (Figs. 4 and 5*C*), it is more likely that the inhibitory effect of the sulfhydryl-binding agent, IAA (Fig. 5, *A* and *B*), may be due to an indirect effect via steric interference to hepcidin binding or conformational changes in α_2M and α_2M-MA after thiol modification. Therefore, the greater decrease in hepcidin binding to α_2M-MA, relative to α_2M, in the presence of IAA (Fig. 5, *A* and *B*), suggests thiols exposed by activation of α_2M (18) are an important component in α_2M-MA·hepcidin binding. Moreover, taking into account the mechanism by which hepcidin interacts with Fpn1 (58), steric constraints are likely to prevent hepcidin internally bound to α_2M/α_2M-MA from interacting with Fpn1. Hence, the labile release of hepcidin α_2M/α_2M-MA appears critical in terms of its ability to mediate down-regulation of Fpn1 (Fig. 9).

Furthermore, it is of significant interest that hepcidin binding to both α_2M and α_2M-MA is markedly pH-dependent (Fig. 5, *C* and *D*). In this case, slight acidification leads to a pronounced reduction in hepcidin binding. This again suggests a labile, noncovalent interaction that can be speculated to have important pathophysiological consequences. In fact, it is of interest that the extracellular fluid in inflammatory sites has been known to be acidic for greater than 60 years (59). Hence, local pH changes during inflammation may affect hepcidin binding to native and activated α_2M. Such an effect may be significant during bacterial infection, where the local hypofer-

remia induced by the release of hepcidin in the proximity of Fpn1 would inhibit cellular iron release. This response could retard extracellular bacterial growth, as iron is essential for replication (60). Moreover, the direct anti-bacterial activity of human hepcidin (61) released from α_2M may also be important for retarding bacterial growth. Clearly, further comprehensive studies are required to directly investigate this hypothesis.

Model of Hepcidin Transport, a Labile Interaction Facilitates Transport of Hepcidin by α_2M and α_2M-MA and Its Release to Fpn1—Previous studies have identified noncovalent, site-specific interactions between α_2M and growth factors/hormones (13, 21–23). These regions could be involved in the binding and labile release of hepcidin and demonstrate that an equilibrium is set up between protein-bound and free hepcidin that could explain urinary hepcidin excretion (2). Moreover, the presence of Fpn1 on the cell surface may drive the equilibrium toward release of hepcidin from its complexation with α_2M or α_2M-MA (see *model* in Fig. 9). Hence, our studies do not support the idea that α_2M and α_2M-MA bind and trap hepcidin, as occurs with the binding of proteases to the internal thioester bond near the α_2M bait region (20).

In agreement with this model, upon comparison of the efficacy of α_2M·hepcidin and α_2M-MA·hepcidin in decreasing Fpn1 expression, it was shown that although the macroglobulin receptor, Lrp1, only recognizes activated α_2M (18), hepcidin bound to both α_2M and α_2M-MA elicited a similar decrease in Fpn1 expression *in vitro* (Figs. 1*C*, 2, 3, 6 (*C* and *D*), and 7*B*). This observation suggested the passive release of hepcidin from the protein could be important in terms of its effect on Fpn1. This was further supported by our studies demonstrating that Lrp1 was not involved in the down-regulation of Fpn1 after incubation with α_2M-MA·hepcidin or α_2M·hepcidin complexes. Our data and model (Fig. 9) are consistent with a labile interaction that facilitates transport of hepcidin by α_2M and α_2M-MA and its release to Fpn1 (Fig. 9). Similar functions have been indicated for other cytokines/hormones that bind to α_2M/α_2M-MA (8, 10, 11, 24, 62).

Hypoferremic Effect of Hepcidin Is Accentuated When Bound to α_2M and Particularly α_2M-MA—Of particular significance, the *in vivo* studies presented herein of serum iron levels demonstrated the hypoferremic effect of hepcidin was accentuated when bound to α_2M and especially α_2M-MA, which could be due to functional enhancement by hepcidin binding to α_2M/α_2M-MA. In these studies, the estimated concentration of hepcidin in mouse blood upon initial injection was ~0.45 μM, which is in the range of patients with inflammation (48). This concentration was used to ensure a clear response that was measurable and relevant to the *in vivo* situation.

However, our *in vitro* data demonstrated that although α_2M·hepcidin/α_2M-MA·hepcidin effectively decreased Fpn1 expression, it was generally less effective than hepcidin at physiologically relevant concentrations (0.01–0.1 μM; Fig. 3) (48). We hypothesize that two factors are responsible for the differences observed between our *in vitro* and *in vivo* experiments. The first factor to be considered is that the binding of hepcidin (~2.8 kDa) to α_2M (~725 kDa) retards glomerular filtration (Fig. 8*D*), thereby increasing its circulatory half-life compared with free hepcidin (Fig. 9). Thus, once hepcidin is bound to α_2M, its prolonged half-life in the circulation leads to an enhanced opportunity to interact with Fpn1 than free hepcidin, resulting in a greater hypoferremic response (see Figs. 8*B* and 9). Relevant to this, it has been previously shown that excess free (nonprotein bound) hepcidin becomes detectable in urine <1 h after *intraperitoneal* injection (47). This may reflect hepcidin excretion that is present in excess of the binding capacity of endogenous murine homologs of α_2M, namely α_2M and murinoglobulin (63). This hypothesis was confirmed in this study in experiments demonstrating that unbound ^{125}I-hepcidin was significantly increased in the kidney (Fig. 8*C*) and urine (Fig. 8*D*) relative to ^{125}I-hepcidin complexed to α_2M.

Another factor that may be responsible for the greater activity *in vivo* of hepcidin bound to α_2M-MA relates to differences in the processing of α_2M-MA·hepcidin by the hepatocyte relative to both free hepcidin or α_2M·hepcidin (which cannot bind to Lrp1 (18)). In contrast to the ability of α_2M·hepcidin/α_2M-MA·hepcidin to down-regulate Fpn1 independently of Lrp1 *in vitro* (Fig. 6, *C* and *D*), α_2M-MA·hepcidin binding to Lrp1 *in vivo* may play a vital role in presenting hepcidin to Fpn1- and Lrp1-rich liver (55, 64). This is suggested by the greater hypoferremic response achieved using complexes of α_2M-MA·hepcidin relative to α_2M·hepcidin (Fig. 8*B*). In this case, the greater uptake of α_2M-MA·hepcidin potentially mediated by hepatic Lrp1 (Fig. 8*C*), and the resulting enhanced hypoferremia (Fig. 8*B*), could be due to an influx of hepcidin delivered as the α_2M-MA·hepcidin complex to the local hepatic environment. This is related to the marked uptake of ^{125}I-α_2M-MA by the liver relative to ^{125}I-α_2M (50) and the significantly greater accumulation of ^{125}I-hepcidin in the liver when bound to α_2M-MA relative to α_2M or free hepcidin (Fig. 8*C*).

In summary, we demonstrate that α_2M and α_2M-MA form complexes with hepcidin which *in vitro* elicit decreased Fpn1 expression independently of the α_2M receptor, Lrp1. We also show that serum iron levels are reduced to a significantly greater extent in mice treated with α_2M·hepcidin and particularly α_2M-MA·hepcidin complexes relative to unbound hepcidin. Hence, this study highlights that hepcidin diagnostic kits should document their ability to measure hepcidin bound to α_2M to quantitatively assess blood hepcidin levels. In fact, currently, protein binding of hepcidin is not taken into account and appears to be important in terms of quantitatively assessing hepcidin in blood. This may explain the wide variation in hepcidin levels observed in international round robin studies aimed at measuring this peptide in blood samples (65).

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