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Characterisation of the Transition Metal Binding Properties of Hepcidin

Running Head; Hepcidin-metal binding

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Synopsis; Accumulating evidence suggests that hepcidin, a 25 residue peptide hormone, is the master regulator of iron metabolism. Further evidence suggests that the five N-terminal amino acids are crucial for mediating its biological function. With a histidine residue at position 3, this region also has the potential to bind divalent metal ions. To characterise this hepcidin-metal interaction in detail this study utilises electrospray mass spectrometry to measure the binding of a range of metal ions to wild type and mutant human and murine hepcidins. In addition the biological effects of these point mutations were tested on Caco-2 and HEK 293T human cell lines and in mice. Our results show that hepcidin-25 can form complexes with copper, nickel and zinc, though we failed to detect any hepcidin-25 binding to either ferric or ferrous ions. The greatest affinity observed was between hepcidin-25 and copper with a dissociation constant $\ll 1 \mu\text{M}$. Substituting the histidine at position 3 in human hepcidin-25 and comparably the asparagine at position 3 in murine hepcidin-25 with alanine markedly diminished the affinity for copper. The amino acid substitutions also decreased the biological activity of hepcidin-25; namely repression of ferroportin protein levels and hypoferraemia. In summary the high affinity of hepcidin for copper suggests that hepcidin could bind copper *in vivo* and this may be of biological relevance.

Keywords; Hepcidin, iron, metal, copper, zinc

INTRODUCTION

Overwhelming evidence has identified the mature 25 amino acid hepatic anti-microbial peptide hepcidin as the master regulator of iron metabolism in man [1,2]. It is now also clear that there are several pathways involved in the regulation of this peptide hormone including inflammation, infection, erythropoietic demand, hypoxia and body iron status [3]. Whilst exactly which intracellular signalling pathways are responsible for regulating hepcidin remains to be unravelled, it is clear that hepcidin mediates its biological activity by interacting with the cellular iron efflux protein ferroportin which is present on most cell types [4]. This hepcidin interaction results in the internalisation and degradation of ferroportin decreasing cellular iron efflux [4,5]. There is also emerging evidence that the process of cellular iron efflux is also likely to be dependent upon the multicopper oxidases ceruloplasmin and hephaestin [6] which are thought to function as ferroxidases [7-9].

The hepcidin gene encodes an 84 amino acid pre-propeptide which is cleaved by furin to generate the biologically active 25 residue peptide (hepcidin-25). Hepcidin forms a distorted β -sheet stabilised by 4 intramolecular disulphide bonds spanning residues 7 to 23 leaving a flexible 6-residue N-terminal region [10,11]. In addition to hepcidin-25, two shorter forms, hepcidin-20 and hepcidin-22 have been identified where the N-terminal 5 and 3 amino acids are missing respectively. These are thought to represent breakdown products of hepcidin-25 [12]. Hepcidin-20 has no effect on cellular iron efflux but does appear *in-vitro* to exert anti-bacterial and anti-fungal properties, albeit at supra physiological concentrations [13,14]. This suggests that the first five amino terminal amino acids of hepcidin are essential for mediating its role in regulating iron metabolism and this has been confirmed by the elegant studies of Ganz and colleagues which showed that sequential N-terminal amino acid deletion resulted in a stepwise loss of ferroportin binding and biological activity with >50% loss upon removal of the first three amino acids (aspartic acid, threonine and histidine) [15,16].

Melino *et al* have shown that the N-terminal region of hepcidin-25 is capable of binding divalent copper and nickel ions via an 'Amino Terminal Cu-Ni' (ATCUN) binding motif comprising the three N-terminal residues [17]. However, this study largely used truncated forms of hepcidin-25 and utilised both high concentrations of hepcidin and metal ion. In addition these studies were unable to provide information on affinity or stoichiometry. Studies by Farnaud *et al* suggest that hepcidin may bind iron, although this interaction was only ever observed under reducing conditions and not with disulphide bonded hepcidin (generally thought to be the biologically active form) [18,19]. These suggestions that hepcidin might bind metal ions, in particular copper, are interesting as disturbances of copper metabolism have been shown to lead to altered iron homeostasis [7-9]. Although these defects are generally thought to be caused by decreased activity of the ferroxidases hephaestin and ceruloplasmin, an interaction between copper and hepcidin had not been considered. Thus the aim of this study is to further characterise metal ion binding to hepcidin and, using mutation analysis, determine the critical amino acids within hepcidin responsible for binding. If hepcidin does bind metal ions with high affinity then this could have major implications in the regulation of human iron metabolism.

EXPERIMENTAL

Materials

All peptides were synthesised by Alta Bioscience (University of Birmingham, UK) and provided as linear (reduced) peptides. These were allowed to spontaneously oxidise by dissolving at 0.1 mg/ml in 6M urea, 30 mM MOPS (pH 7.0) and incubating overnight at room temperature with stirring as previously described (20). Complete disulphide bridge formation was accompanied by an 8 Dalton decrease in mass in MALDI-TOF mass spectra. The folded hepcidin was purified by C18 RP-HPLC in 0.1% TFA/acetonitrile and lyophilised. Hepcidin concentrations were determined by BCA assay calibrated with bovine serum albumin (Pierce). The sequences of the synthetic hepcidin peptides are as follows with amino acid substitutions underlined;

Human hepcidin-25: DTHFPICIFCCGCCHRSKCGMCCKT

Human H3A hepcidin: DTAFPICIFCCGCCHRSKCGMCCKT

Human hepcidin-20: ICIFCCGCCHRSKCGMCCKT

Murine hepcidin-25: DTNFPICIFCCCKCCNNSQCGICCKT

Murine N3A hepcidin: DTAFPICIFCCCKCCNNSQCGICCKT

Hepcidin-25 was purified from urine after informed consent had been obtained from patients as approved by the ethics research committee of Innsbruck Medical University (protocol Nr. UN3479). The purification was based on the method of Park *et al* [14]. Briefly, urine was centrifuged at 3000 g for 30 min, passed through a 0.45 µm filter and diluted 1:10 with water before loading onto a 50 ml weak cation exchange column (GE Healthcare, Vienna, Austria). After washing with 5 column volumes of 25 mM ammonium acetate, hepcidin was eluted with 5 % acetic acid. The eluate was lyophilized, dissolved in 0.1 % TFA and applied to a 10 × 250 mm C18 column (218TP510, Grace Vydac, USA) equilibrated in 0.1 % TFA, 2 % acetonitrile (ACN). Peptides were separated with an acetonitrile gradient of 3.6 % ACN increment/min for 5 min followed by a 1 % ACN increment for 25 min and a 3.5 %/min increase over 10 minutes followed by a 4 %/min increment for the final 5 min of the run at a flow rate of 1.5 ml/min. Hepcidin elution was monitored by MALDI-TOF mass spectrometry.

Hepcidin Mass Spectrometry

Mass spectra were acquired on a 7-T LTQ FT instrument (Thermo Fisher Scientific, Germany). Peptides were dissolved in 10 mM ammonium acetate, pH 6.8, and directly infused into the mass spectrometer at a flow rate of ~ 300 nL min⁻¹ using a Triversa Nanospray source (Advion Biosciences, NY). The nanoelectrospray voltage was +1.7 kV. Data was collected for 1 minute per sample. The fraction of hepcidin with metal ion bound was estimated from the relative peak heights of the triply charged ions of free hepcidin and hepcidin-metal complex. Where peaks corresponding to hepcidin with 1, 2 or 3 metal ions bound were present the heights of all 3 were added together to calculate the fraction of 'complex' i.e. hepcidin-metal complex containing at least one metal ion.

Effect of hepcidin on ferroportin expression in Caco-2 cells

The cell line Caco-2 was routinely cultured in Dulbecco's modified eagles medium (Gibco, USA) with 10% foetal calf serum supplemented with 100 units/ml penicillin and 0.1mg/ml streptomycin. Upon reaching 14 days post confluence cells were challenged with either growth medium alone (control) or with i) human hepcidin-25 peptide, ii) human H3A hepcidin-25 or iii) hepcidin-20

supplementation at concentrations of 1 μ M. Following 48hrs stimulation cells were lysed and processed for Western blotting with a rabbit polyclonal antibody to ferroportin (1000 dilution; Alpha Diagnostics Inc., San Antonio, TX1) and a mouse monoclonal β -actin antibody (1:5,000 dilution; Abcam ab8226-100); the latter was employed for normalisation of protein loading. All immunoreactive bands were then subject to densitometry using NIH Image 1.62 software.

Effect of hepcidin in a ferroportin-GFP assay

HEK 293T cells (2×10^5) were seeded in six well plates 24 hrs prior to transfection. Cells were then transiently transfected with the wild-type FPN-GFP plasmid construct pBABS, (created by recombination of the pENTR221 clone IOH26826 (Invitrogen, Lofer, Austria) containing the wild-type human ferroportin ORF with the vector pcDNA6.2/N-EmGFP-DEST (Invitrogen)), using the liposomal transfection reagent Effectene (Qiagen) following the manufacturer's instructions. After 16 hours transient transfection, cells were then incubated in the presence or absence of 0.5 μ M hepcidin for a further 16 hours. For flow cytometry cells were detached from the culture plates with cell dissociation solution (Sigma, Vienna, Austria) and gated for GFP expression using untransfected cells as negative control. Hepcidin induced ferroportin GFP degradation is expressed as mean fluorescence of 50,000 cells expressed as a ratio of mean fluorescence in hepcidin treated vs. untreated FPN-GFP expressing cells (control).

Animal model for testing the effect of hepcidin peptides

6 week old CD1 male mice (weights 28-32g) (Charles Rivers, Margate, Kent), were subject to intra-peritoneal injection with either i) 0.15M NaCl (control) ii) murine hepcidin-25 or iii) murine N3A hepcidin-25 (25 μ g/mouse). At 4 hrs post injection mice were anaesthetised, sacrificed by cervical dislocation and blood collected by cardiac puncture. Serum iron analysis was performed using a serum iron assay kit (Thermo Electron Corporation, Louisville, USA) according to the manufacturers' instruction. All animal experiments were performed under the authority of a UK Home Office licence. Mice were fed RM1 diet (Scientific Diet Supplies [SDS], Witham, Essex, United Kingdom). Experiments were performed in triplicate.

RESULTS

Hepcidin mass spectrometry: The monoisotopic m/z value of the triply charged ion at 930.023 is the most abundant signal generated from synthetic human hepcidin-25 sprayed into the mass spectrometer at 5 μ M (Figure 1A). Addition of sub-stoichiometric CuSO₄ generates a peak corresponding to the triply charged hepcidin-copper complex at m/z 950.329 (Figure 1B). The m/z change between the monoisotopic triply charged peaks of the free hepcidin and hepcidin-copper complex equates to the complex being 60.9 Daltons heavier than the free hepcidin. The most abundant isotope of copper (69%) has an atomic mass of 62.9. Thus there is a loss of 2 Daltons and 2 positive charges when the complex is formed indicating that 2 H⁺ ions are lost during complex formation. Addition of equimolar copper to the hepcidin-25 is sufficient for all of the hepcidin to exist as the hepcidin-copper complex (Figure 1C).

Copper binding to human hepcidin: To further characterise copper binding to human hepcidin, mass spectra were obtained using 5 μ M hepcidin and 0-50 μ M CuSO₄ (Figure 2A). Human hepcidin-25 binds copper with an affinity such that at 5 μ M hepcidin and 5 μ M Cu²⁺ no free hepcidin is detectable. In contrast only ~20 % of hepcidin-20 exists as a copper complex at 50 μ M Cu²⁺ clearly demonstrating that the high-affinity copper binding requires the presence of the N-

terminal 5 residues of hepcidin. Changing the histidine residue at position 3 to an alanine residue also reduces the affinity of hepcidin for copper with 15-20 μM Cu^{2+} being required for 50% complex formation with H3A hepcidin. The very high-affinity of hepcidin-25 for copper was further explored by decreasing the hepcidin concentration to 1 μM and adding copper in 0.2 μM increments (Figure 2B). Stoichiometric copper was sufficient for complete complex formation (in several independent repeats saturation was obtained at 0.8 Cu^{2+} per hepcidin suggesting an overestimation of the hepcidin concentration by ~25%). This experiment indicates that the dissociation constant for Cu^{2+} binding to hepcidin-25 is well below 1 μM . A more accurate estimate is difficult to obtain as the mass spectra became unreliable below 1 μM hepcidin.

Copper binding to murine hepcidin-25 and mutant N3A hepcidin: The affinity of mouse hepcidin for copper is substantially lower than that of human hepcidin with ~8 μM CuSO_4 required for 50% complex formation. (Figure 3) As with human hepcidin the residue at position 3 appears to be involved in the binding as when this asparagine is replaced with alanine there is an approximate 5-fold decrease in the affinity for Cu^{2+} affinity (~40 μM Cu^{2+} required for 50% complex formation with N3A hepcidin) (Figure 3).

Binding of other transition metal ions to human hepcidin: We tested the ability of 200 μM iron (FeSO_4 , Fe^{2+} and FeCl_3 , Fe^{3+}), zinc (ZnSO_4 , Zn^{2+}) and nickel (NiSO_4 , Ni^{2+}) to form complexes with 5 μM human hepcidin-25. Neither Fe^{2+} nor Fe^{3+} showed any evidence of complex formation, whereas Zn^{2+} , and Ni^{2+} both formed complexes with hepcidin. Ni^{2+} binds with much lower affinity than copper (K_d of ~150 μM) and Zn^{2+} with lower affinity still (Figure 4). Hepcidin did not appear to bind more than one Zn^{2+} or Ni^{2+} ion whereas at 200 μM Cu^{2+} hepcidin-metal complexes containing 2 or 3 metal ions were detectable (data not shown).

Metal binding to urinary hepcidin: The most intense signal in the mass spectrum of hepcidin-25 purified from human urine was a triply charged ion at m/z 930.022 i.e. identical to that of synthetic hepcidin-25. No hepcidin-copper complex was observed in the absence of exogenous CuSO_4 addition. This is as expected because the hepcidin purification included HPLC in acid conditions which would have removed any bound metal ions. As with the synthetic peptide, addition of stoichiometric CuSO_4 resulted in the disappearance of the hepcidin peak and the appearance of a peak corresponding to triply charged hepcidin-copper complex at m/z 950.325. The theoretical and experimentally determined masses of all the hepcidin peptides used in this study and their copper complexes are presented in Table 1. Theoretical masses were calculated from published hepcidin sequences and adjusted for the 4 internal disulphide bonds present in folded hepcidin [10,11]. The calculated isotopic distribution (based on the empirical formula of hepcidin including 4 disulphide bonds) and measured isotopic distribution for hepcidin-25 and hepcidin-25 complexed with copper are shown in Figure 5. The close agreement between theoretical and measured m/z values and isotopic distributions demonstrate that we are measuring monomeric hepcidin with or without a single metal ion bound. We also used collision induced dissociation to obtain MS/MS spectra of both free hepcidin and the hepcidin-copper complex. Similar fragmentation patterns were obtained in both cases although in the complex the y22 ion (residues 4-25) predominated whereas the y21 ion (residues 5-25) was the most intense fragment of free hepcidin (data not shown). Titration of 1 μM urinary hepcidin with increasing concentrations of CuSO_4 (Figure 6) produced a binding curve very similar to that obtained with

synthetic hepcidin (Figure 3) showing near complete binding of Cu^{2+} at all CuSO_4 concentrations. This shows that native human hepcidin can bind Cu^{2+} with a dissociation constant $\ll 1 \mu\text{M}$.

Effect of hepcidin peptides on ferroportin expression in Caco-2 and HEK293T cells: To determine if substitution of histidine for alanine influenced the biological activity of hepcidin the intestinal cell line Caco-2 was challenged with hepcidin-25, H3A hepcidin-25 or hepcidin-20 followed by determination of ferroportin levels by Western blotting. In agreement with previous studies, challenging with hepcidin-25 but not hepcidin-20 caused a decrease in ferroportin levels ($P < 0.05$). However, substitution of histidine in position 3 for alanine (H3A hepcidin-25) abrogated the ability of hepcidin to decrease ferroportin expression (Figure 7A-B). In support of this observation utilising the well characterised HEK-293T ferroportin-GFP assay we observed a statistically significant ($P < 0.05$) decrease in GFP fluorescence with both human urinary purified hepcidin-25 and synthetic hepcidin 25 but not with H3A hepcidin-25 (Figure 7C).

Effect of hepcidin peptides on murine serum iron levels: To determine the biological importance of asparagine at position 3 in murine hepcidin-25 both wild type and N3A hepcidins were injected into wild type CD1 male mice (Figure 8). As expected wild type hepcidin caused a significant hypoferraemic effect after 4hrs compared to control mice that received saline alone ($p < 0.05$). Injecting mice with the murine N3A hepcidin-25 peptide did not induce a significant hypoferraemia.

DISCUSSION

We have used electrospray FTICR mass spectrometry to characterise the binding of several transition metal ions to hepcidin. This technique has previously been used to study metal ion binding to peptides and small proteins [21,22] and the use of non-denaturing conditions has allowed us to investigate the specificity, affinity and stoichiometry of metal ion binding to hepcidin. Hepcidin-metal complexes were observed with copper, nickel and zinc with a gradient of highest to lowest affinity as follows $\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+}$. Hepcidin binds copper with a dissociation constant $\ll 1 \mu\text{M}$. Our data is consistent with previous work in terms of the metal specificity and the affinity of the ATCUN motif for copper [21,23]. Indeed, the pioneering work of Lau *et al* [23] demonstrated that both the ATCUN motif in albumin and also a short synthetic peptide containing an ATCUN motif bind copper with sub-femtomolar dissociation constants.

Most of our data were obtained using synthetic hepcidin, however, as there has been controversy with regards the structure of hepcidin [15,16] and whether synthetic hepcidin and 'native' hepcidin have identical properties we examined this metal-hepcidin interaction with hepcidin purified from human urine. Results presented herein show that hepcidin purified from human urine can bind copper with high affinity excluding the possibility that such an interaction is only a property of synthetic hepcidin. However, using synthetic hepcidin allowed us to make several variants of hepcidin to further characterise copper binding. Hepcidin-20 showed vastly reduced copper binding indicating that the high affinity site resides within residues 1-5. Moreover, and as expected, the histidine at position 3 plays a crucial role and substituting this with alanine greatly decreases the affinity of hepcidin for copper. The sequence of hepcidin is highly conserved across 23 mammalian species and the N-terminal region is particularly highly conserved. The entire

sequence is identical in 9 primates but the N-terminal 6 residues are identical in all the mammalian hepcidins except rodents (rat and mouse) where histidine 3 is replaced by asparagine. We find that this amino acid substitution results in greatly reduced copper affinity although the asparagine does support some copper binding as evidenced by the further decrease in copper affinity when the asparagine is replaced with alanine.

Introducing alanine at position 3 in human hepcidin not only decreases metal binding but also biological activity as evidenced by decreased ferroportin expression in both Caco-2 and HEK-293T cells. With respect to the murine hepcidin data, we again demonstrate decreased copper binding upon substitution of asparagine at position 3 with alanine, and this amino acid change also appears to decrease biological activity, as evidenced by a lack of a hypoferraemic response in mice. However, we cannot exclude the possibility that the mutant hepcidin retains some biological activity not detected under these experimental conditions. Furthermore, detailed studies would be required to determine whether the loss of metal binding is responsible for this effect, whether the amino acid substitutions cause changes in the structural/biophysical properties of hepcidin or whether the presence of the bulky histidine/asparagine side chain plays a crucial role in determining biological activity.

The data presented in this study support the report of Melino *et al* where hepcidin-metal binding (copper and nickel) was observed using spectroscopic methods [17]. However, we failed to demonstrate any interaction between hepcidin and ferric or ferrous iron as previously reported by Farnaud *et al* [18-19]. We speculate that this is due to a difference in the form of hepcidin used in the studies, with our study using the biologically active fully folded form of hepcidin as opposed to the reduced linear form of hepcidin used by Farnaud *et al*.

Given the affinities determined in this study and the circulating levels of the various transition metals in human serum, the only interaction which is likely to be physiologically relevant is that of copper binding to the N-terminal region of hepcidin. The vast majority of plasma copper is in complex with ceruloplasmin, however, free copper may be as high as 0.2 μM in healthy individuals and elevated to 1 μM in patients with Wilson's disease [24] i.e. high enough to promote complex formation with hepcidin. The residues comprising the copper binding site are also crucial to hepcidin's ability to regulate iron metabolism [15,16]. It is likely that copper binds to this region of hepcidin in a similar manner to other reported ATCUN-copper interactions [23]: coordination of the metal ion by the N-terminal amine group, the amide groups of residues 2 and 3 and the imidazole group of the histidine. The resulting conformational restraints on the N-terminal region of hepcidin (which appears to be disordered in NMR and crystal structures [10,11]) would alter its ability to bind to ferroportin and/or other *in vivo* binding partners.

In summary, we have shown that hepcidin has a very high affinity for copper ions *in vitro*. We also show that the amino acid residues essential for copper binding are also essential for biological activity. Further work is required to investigate whether copper-hepcidin binding plays a role in iron and/or copper homeostasis.

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Table 1.

Peptide	Mass calculated from sequence (Da)	m/z of 3 ⁺ ion calculated from sequence	Experimentally determined m/z of 3 ⁺ ion	Mass calculated from experimental data (Da)
Human hep-25 <u>D</u> <u>T</u> H <u>F</u> PICIFCCGCCHRSKCGM <u>C</u> <u>C</u> <u>K</u> <u>T</u>	2787.025	930.016	930.023	2787.047
Human hep-25 +copper	2847.940	950.321	950.329	2847.965
Human hep-20 ICIF* <u>C</u> <u>C</u> <u>G</u> <u>C</u> <u>C</u> <u>H</u> <u>R</u> <u>S</u> <u>K</u> <u>C</u> <u>G</u> <u>M</u> <u>C</u> <u>C</u> <u>K</u> <u>T</u>	2199.857	734.293	734.277	2199.809
Human hep-20 +copper	2260.772	754.598	754.582	2260.724
Human H3A <u>D</u> <u>T</u> <u>A</u> <u>F</u> <u>P</u> <u>I</u> <u>C</u> <u>I</u> <u>F</u> <u>C</u> <u>C</u> <u>G</u> <u>C</u> <u>C</u> <u>H</u> <u>R</u> <u>S</u> <u>K</u> <u>C</u> <u>G</u> <u>M</u> <u>C</u> <u>C</u> <u>K</u> <u>T</u>	2721.003	908.008	908.015	2721.023
Human H3A +copper	2781.918	928.313	928.318	2781.932
Mouse hep-25 <u>D</u> <u>T</u> <u>N</u> <u>F</u> <u>P</u> <u>I</u> <u>C</u> <u>I</u> <u>F</u> <u>C</u> <u>C</u> <u>K</u> <u>C</u> <u>C</u> <u>N</u> <u>N</u> <u>S</u> <u>Q</u> <u>C</u> <u>G</u> <u>I</u> <u>C</u> <u>C</u> <u>K</u>	2752.015	918.346	918.348	2752.022
Mouse hep-25 +copper	2812.930	938.651	938.652	2812.934
Mouse N3A <u>D</u> <u>T</u> <u>A</u> <u>F</u> <u>P</u> <u>I</u> <u>C</u> <u>I</u> <u>F</u> <u>C</u> <u>C</u> <u>K</u> <u>C</u> <u>C</u> <u>N</u> <u>N</u> <u>S</u> <u>Q</u> <u>C</u> <u>G</u> <u>I</u> <u>C</u> <u>C</u> <u>K</u> <u>T</u>	2709.010	904.010	904.013	2709.017
Mouse N3A +copper	2769.925	924.315	924.319	2769.935
Hep-25 (human urine) <u>D</u> <u>T</u> H <u>F</u> PICIFCCGCCHRSKCGM <u>C</u> <u>C</u> <u>K</u> <u>T</u>	2787.025	930.016	930.022	2787.044
Hep-25 (human urine) + copper	2847.940	950.321	950.325	2847.953

Table 1. **Theoretical and measured masses of hepcidin peptides and their complexes with copper.** We show the monoisotopic masses calculated from the amino acid sequence and the predicted m/z ratios of the triply charged ions of these peptides. All calculated masses include adjustment for the presence of 4 internal disulphide bonds. The calculated masses for the copper complexes include loss of two H⁺ ions during complex formation. We also show the m/z ratios determined by FTICR mass spectrometry and the monoisotopic molecular masses calculated from these. The sequences of the hepcidin peptides are shown with amino acid substitutions underlined. The hepcidin-20 used in this study incorporated a stable isotope labeled phenylalanine residue indicated by an asterisk (plus 10 Da).

Figure 1

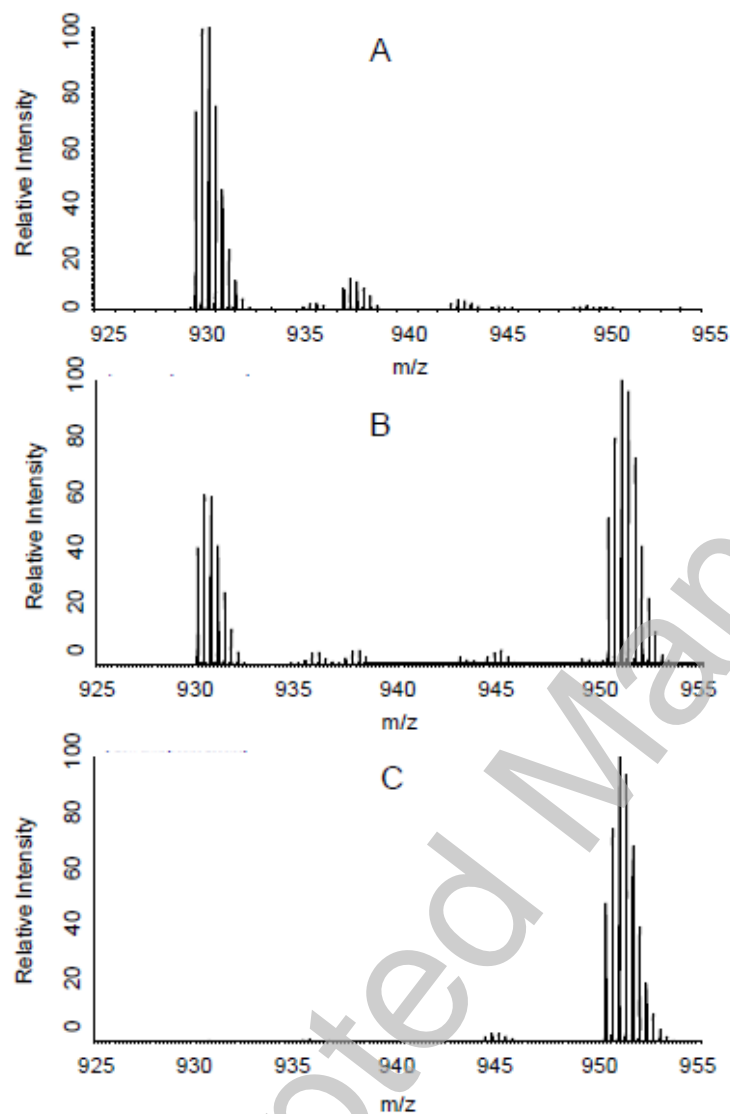


Figure 1. FTICR mass spectra of human hepcidin-25 and hepcidin-copper complexes.

A; In the absence of CuSO_4 only the triply charged ion of free hepcidin (m/z 930.02) is observed. *B*; Addition of $2 \mu\text{M}$ CuSO_4 results in both the triply charged ions of free hepcidin and hepcidin-copper complex (m/z 950.33). *C*; Upon adding $5 \mu\text{M}$ CuSO_4 the only peak observed is that corresponding to the hepcidin metal complex.

Figure 2

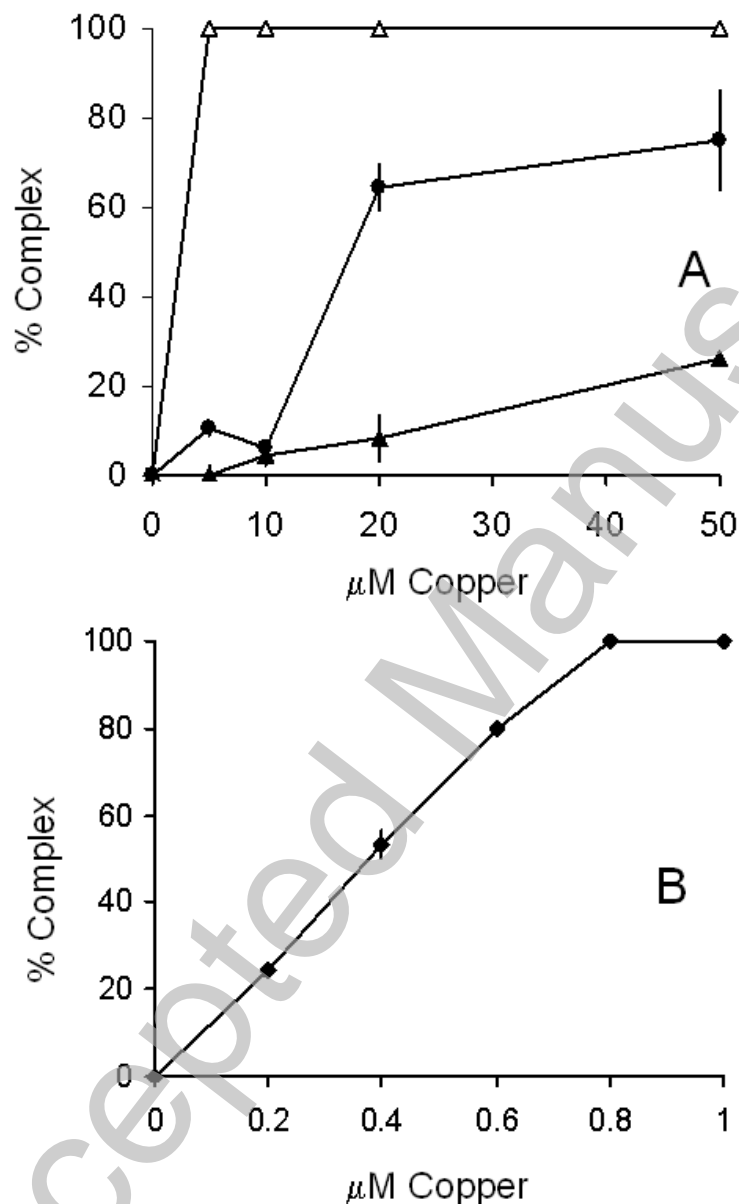


Figure 2. Cu^{2+} binding to wild type and mutated human hepcidin.

A; In separate experiments, 5 μM human hepcidin-25 (open triangles), hepcidin-20 (filled triangles) and H3A hepcidin (filled circles) were titrated with CuSO_4 and hepcidin-copper complex formation determined by mass spectrometry. *B*; The very high-affinity of hepcidin-25 for copper was further explored by decreasing the hepcidin concentration to 1 μM and adding copper in 0.2 μM increments.

Figure 3

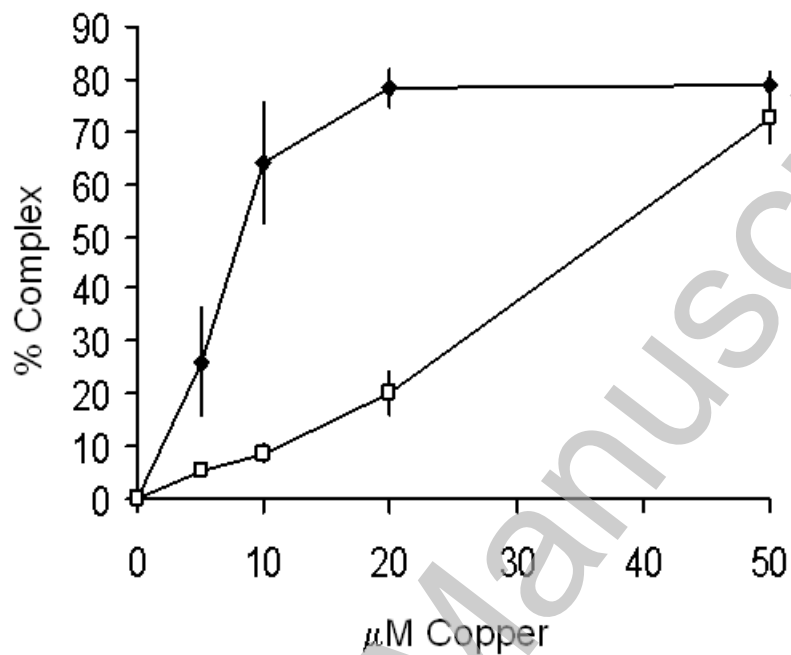
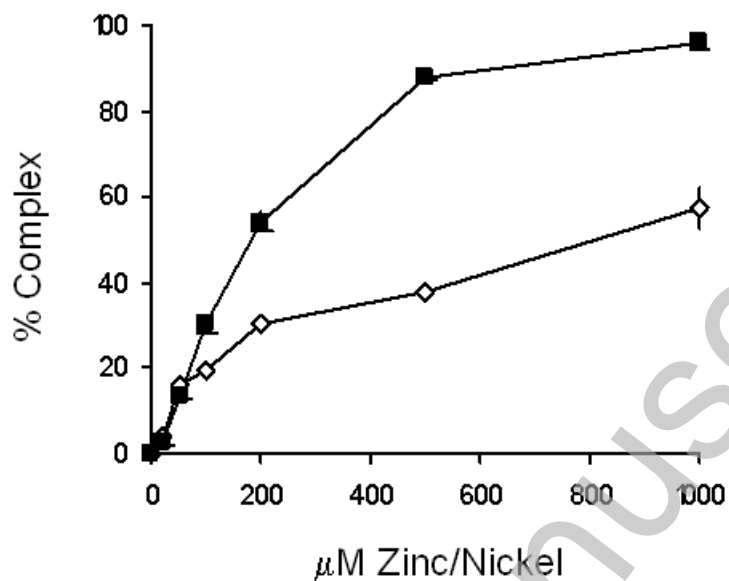


Figure 3. Cu^{2+} binding to murine hepcidin.

5 μM mouse hepcidin-25 (filled diamonds) and N3A mouse hepcidin (open squares) were titrated with CuSO_4 and hepcidin-copper complex formation determined by mass spectrometry.

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Figure 4

**Figure 4. Ni²⁺ and Zn²⁺ binding to human hepcidin**

Human hepcidin-25 (5 μM) was titrated with NiSO₄ (filled squares) or ZnSO₄ (open diamonds) as indicated and complex formation determined by mass spectrometry

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Figure 5

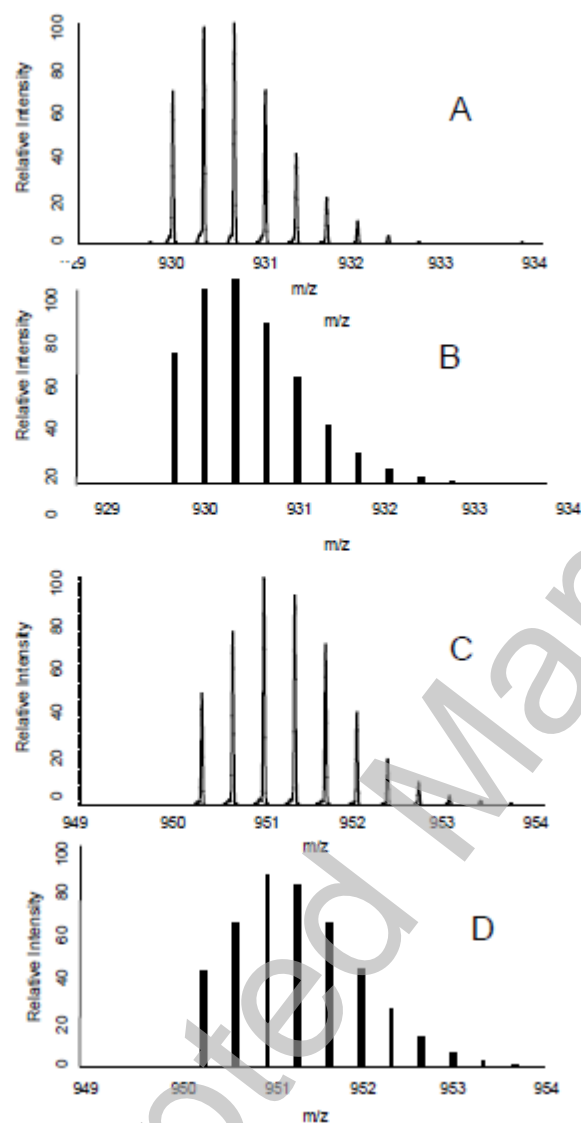


Figure 5. Isotopic distributions of hepcidin and hepcidin-copper complex.

A; the isotopic distribution of the triply charged ion of synthetic human hepcidin-25. *B*; the expected isotopic distribution calculated from the empirical formula of hepcidin-25 plotted on the same axes. *C*; the triply charged ion of synthetic human hepcidin-25 copper complex and *D*; the expected isotopic distribution with the multiple isotopes of copper broadening the isotopic distribution.

Figure 6

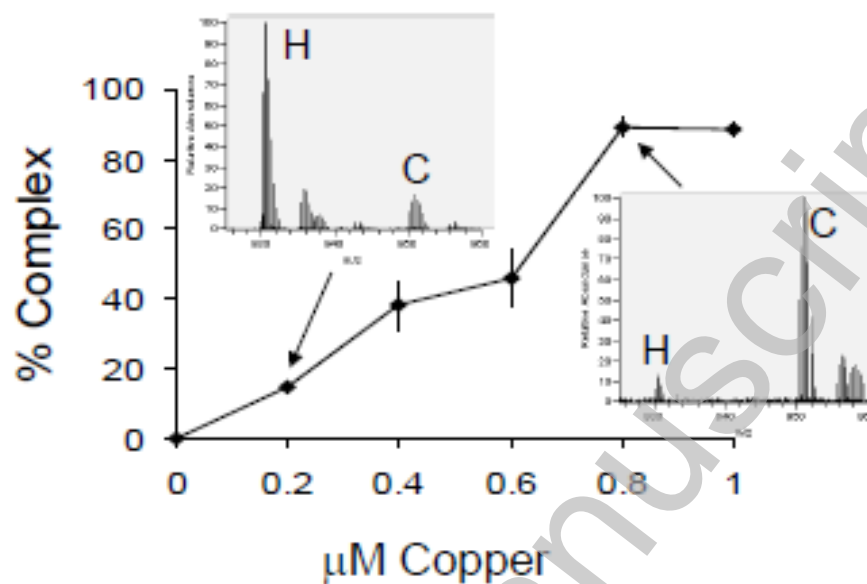


Figure 6. Cu^{2+} binding to $1\mu\text{M}$ hepcidin purified from human urine.

Urinary hepcidin-25 ($1\mu\text{M}$) was titrated with CuSO_4 and hepcidin-copper complex formation determined by mass spectrometry. The two mass spectra show the relative proportions of free-hepcidin (H) and hepcidin-copper complex (C) upon addition of $0.2\mu\text{M}$ and $0.8\mu\text{M}$ CuSO_4 .

Figure 7

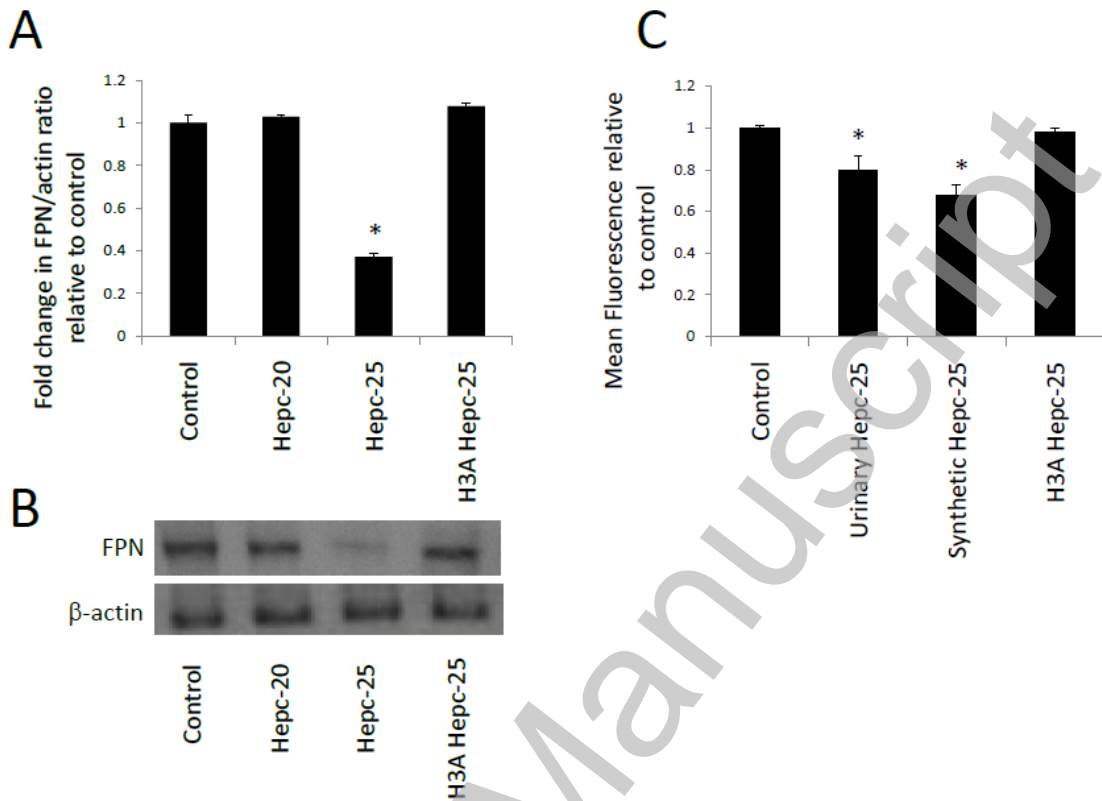


Figure 7. Effect of human hepcidin peptides on ferroportin in Caco-2 and HEK293T cells.

A. Caco-2 cells were challenged with either growth medium alone (control), or human hepcidin-20 (Hepc-20), hepcidin-25 (Hepc-25) or H3A hepcidin-25 (H3A Hepc-25). Cells were then lysed and subject to western blotting for determination of ferroportin (FPN) and β -actin levels; the latter for purpose of normalization. Data is presented as a fold change in the ratio of FPN/ β -actin relative to control cells normalised to 1. B. A representative Western blot for ferroportin and β -actin. C HEK-293T cells were transiently transfected with Ferroportin-GFP followed by challenge with either urinary hepcidin-25, synthetic hepcidin-25 or H3A hepcidin-25 for 16hrs. Cells were then prepared for flow cytometry for GFP expression. Hepcidin induced ferroportin GFP degradation is expressed as mean fluorescence of 50,000 cells expressed as a ratio of mean fluorescence in hepcidin treated vs. untreated FPN-GFP expressing cells (Control; normalised to 1). Error bars denote 1 standard error of the mean. * $p < 0.05$

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Figure 8

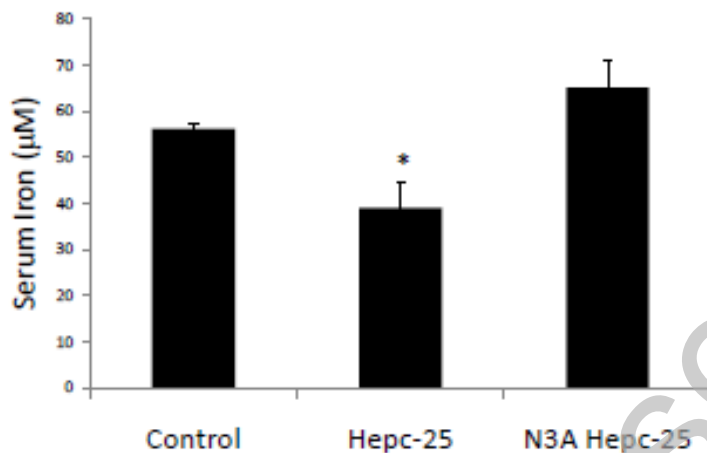


Figure 8. Effect of murine hepcidin peptides on murine serum iron levels.

Peptides corresponding to hepcidin-25 (Hepc-25) and N3A hepcidin-25 were injected into wild type CD1 male mice and after 4hrs serum iron levels were assessed relative to mice injected with saline alone (control). * $p < 0.05$, Student's t-test.