The molecular basis of ferroportin-linked hemochromatosis

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Mutations in the iron exporter ferroportin (Fpn) (IREG1, *SLC40A1*, and MTP1) result in hemochromatosis type IV, a disorder with a dominant genetic pattern of inheritance and heterogeneous clinical presentation. Most patients develop iron loading of Kupffer cells with relatively low saturation of plasma transferrin, but others present with high transferrin saturation and iron-loaded hepatocytes. We show that known human mutations introduced into mouse Fpn-GFP generate proteins that either are defective in cell surface localization or have a decreased ability to be internalized and degraded in response to hepcidin. Studies using coimmunoprecipitation of epitope-tagged Fpn and size-exclusion chromatography demonstrated that Fpn is multimeric. Both WT and mutant Fpn participate in the multimer, and mutant Fpn can affect the localization of WT Fpn, its stability, and its response to hepcidin. The behavior of mutant Fpn in cell culture and the ability of mutant Fpn to act as a dominant negative explain the dominant inheritance of the disease as well as the different patient phenotypes.

hepcidin | iron overload | ferritin | iron export

Hepcidin is a peptide produced by hepatocytes in response to inflammation and iron load, and its expression is suppressed by anemia and hypoxia (1, 2). Hepcidin expression is probably transcriptionally regulated (3, 4), although the exact mechanisms are not completely defined. The circulating peptide acts as the master regulator of cellular iron export by controlling the concentration of ferroportin (Fpn), an iron exporter present on the basolateral surface of intestinal enterocytes and placental cells and on macrophages and hepatocytes (5–7). Hepcidin binds to Fpn and induces its internalization and degradation, resulting in cellular iron retention and decreased iron export (8). Most of the genetic iron overload disorders (hereditary hemochromatosis, HH) result from inadequate hepcidin production relative to the body iron load, thereby permitting excessive duodenal absorption of iron through enterocytes with high concentrations of basolateral Fpn (9–11).

One genetic form of HH, however, does not result from deficient hepcidin production but from mutations in Fpn. In contrast to other forms of HH, type IV HH ("ferroportin disease"), is inherited dominantly (for review see refs. 12 and 13). Notably, all reported mutations in Fpn have been missense mutations that lead to amino acid substitutions or deletions, and no nonsense mutations have been found. The phenotypic manifestations can be classified into two groups, varying in both the severity of tissue iron loading and in the type of tissue affected. One group is characterized by an early rise in ferritin levels with low to normal transferrin saturation and iron accumulation predominantly in macrophages (12). The other group is similar to classical hemochromatosis, with high transferrin saturation and prominent parenchymal iron loading (14). Here, we provide an explanation for the phenotypes and genetic transmission seen in Fpn disease. We show that Fpn is multimeric and that mutant Fpn can multimerize with normal Fpn and affect its function. Depending on the mutation, mutant Fpn can affect the cellular location of WT Fpn and/or its responsiveness to hepcidin.

Methods

Cells and Media. A mouse Fpn cDNA was cloned into the XhoI sites of a CMV-containing vector (pEGFP-N1 from Clontech or pCMV-Tag4 (FLAG) from Stratagene). HEK293T, Cos7, or HeLa cells were maintained in DMEM with 10% FBS and transfected with pFpn-EGFP-N1, pFpn(mutations)-EGFP-N1, or pCMV-Fpn-FLAG by using nucleofector technology (Amaxa, Gaithersburg, MD), according to the manufacturer's instructions. HEK293 Fpn, a stable cell line in which Fpn-GFP is regulated by the ecdysone promoter, is described in ref. 8.

Generation of Fpn Constructs. All human Fpn mutations were generated in pFpn-EGFP-N1 by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) amplified in *Escherichia coli* and sequence-verified before transfecting into mammalian cells.

Other Procedures. Hepcidin was synthesized, iodinated, and used in binding assays as described in ref. 8. Immunofluorescence was performed as described in ref. 8. Transferrin receptor (Tf-R) was detected by using mouse anti-human Tf-R (1:100, no. ab9179-100, Abcam, Cambridge, MA) followed by Alexa Fluor 594-conjugated goat anti-mouse IgG (1:750, Molecular Probes). The mouse anti-FLAG antibody M2 (Sigma) was used to detect Fpn-FLAG by immunofluorescence (1:750) followed by Alexa Fluor 594-conjugated goat anti-mouse IgG (1:750, Molecular Probes). Western blot analysis was performed as described previously by using mouse anti-FLAG antibody (1:10,000, Sigma), rabbit anti-GFP (1:10,000, Abcam no. ab6556), goat anti-human actin (1:1,000), or mouse anti-human EGF receptor (1:1.000, NeoMarkers, Fremont, CA) followed by peroxidase-conjugated goat anti-mouse IgG (1:12,500, Jackson ImmunoResearch), peroxidase-conjugated goat anti-rabbit IgG (1:12,500, Jackson ImmunoResearch), or peroxidase-conjugated donkey anti-goat IgG (1:5,000, Santa Cruz Biotechnology). Ferritin analysis was performed as described in ref. 8. All Western blots were normalized for the total protein concentration by using the bicinchoninic acid assay (Pierce). Immunoprecipitations of Fpn-GFP or Fpn-FLAG were performed by using described protocols (8) or anti-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions. For size-exclusion chromatography, cellular protein was extracted with 150 mM NaCl/10 mM EDTA/10 mM Tris, pH 7.4/1% Triton X-100/protease inhibitor mixture (Roche Applied Science, Indianapolis). One milliliter of Fpn-GFP extract was loaded on a Superdex 200 FPLC column (Amersham Pharmacia) that was standardized by using thyroglobulin (330 kDa), alcohol dehydrogenase (150 kDa), BSA (67 kDa), ovalbumin (40 kDa), and cytochrome *c* (12.4 kDa, Sigma). One-milliliter fractions were collected and analyzed for Fpn-GFP by SDS/PAGE and Western blot.

Abbreviations: Fpn, ferroportin; HH, hereditary hemochromatosis; Tf-R, transferrin receptor.

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Table 1. Summary of the molecular characteristics of disease-inducing Fpn mutations with their respective clinical phenotypes

Fpn-GFP	Reference	Localization	Iron efflux	Hepcidin binding	Hepcidin-induced degradation	Hepcidin-induced iron retention	Clinical phenotype
WT	—	PM	++++	++++	++++	++++	nl
N144H	17	PM	++++	+++	0	0	H
D157G	16	PM (low expression)	++	0	0	0	K
Δ162	22–25	Intracellular/PM	+	0	0	0	K
Δ160–162	This study	Intracellular	+	0	0	0	N.D.
Q182H	16	PM	++++	++++	+ (4 h) +++ (24 h)	+++	K
G323V	16	Intracellular/PM	+	0	0	0	K
G490D	26	Intracellular/PM	+	0	0	0	K

K, iron loading predominantly in Kupffer cells with low transferrin saturation; H, iron loading in hepatocytes with high transferrin saturation; nl, normal; PM, plasma membrane; —, no reference; N.D., no data; +, 25%.

Results

Subcellular Localization of Mutant Fpn. We used site-specific mutagenesis to generate mutations in mouse Fpn-GFP identical to those found in patients with HH type IV (Table 1). The amino acids that were mutated are identical in WT mouse and human Fpn. Cultured HEK293T cells were transfected with plasmids containing mutant Fpn-GFP under the control of the CMV promoter, and the cellular distribution of the expressed protein was examined by fluorescence. Expression of WT Fpn resulted in cell surface localization (Fig. 1*A*, Fpn-GFP). Mutation Fpn(D157G)-GFP is found at the plasma membrane, although the expression of the protein is markedly lower than WT. Some mutations [G323V and G490D (data not shown)] resulted in predominantly intracellular localization, although some protein was found on the cell surface. A mutation that leads to deletion of amino acids 160-162 that was generated in our laboratory resulted in a protein that was entirely intracellular, with no cell surface expression [Fig. 1*A*, Fpn(Δ160-162)-GFP]. One mutant, Fpn(D157G)-GFP, showed significantly reduced expression, although most of the expressed protein localized normally to the cell surface (data not shown). Mutants N144H [Fig. 1*A*, Fpn(N144H)-GFP] and Q182H (data not shown) showed normal cell surface localization. Similar subcellular distributions were seen when the mutant proteins were expressed in Cos7 and HeLa cells (data not shown).

Response of Mutant Fpn to Hepcidin. Addition of hepcidin to cells expressing WT Fpn results in the internalization of cell surface Fpn and its degradation in lysosomes (8). All of the Fpn mutants showed an abnormal response to hepcidin: Internalization of Fpn was either reduced or did not occur. In a 4-h incubation with hepcidin, most of the WT Fpn was internalized, whereas mutant N144H and Q182H Fpn remained on the cell surface (Fig. 1*B*). After a 24-h incubation with hepcidin, most of the mutant Q182H Fpn had been internalized, but N144H remained on the cell surface (Fig. 1*C*). Similarly, mutant D157G remained at the plasma membrane (data not shown). Mutants Δ162, Δ160-162, G323V, and G490D, which

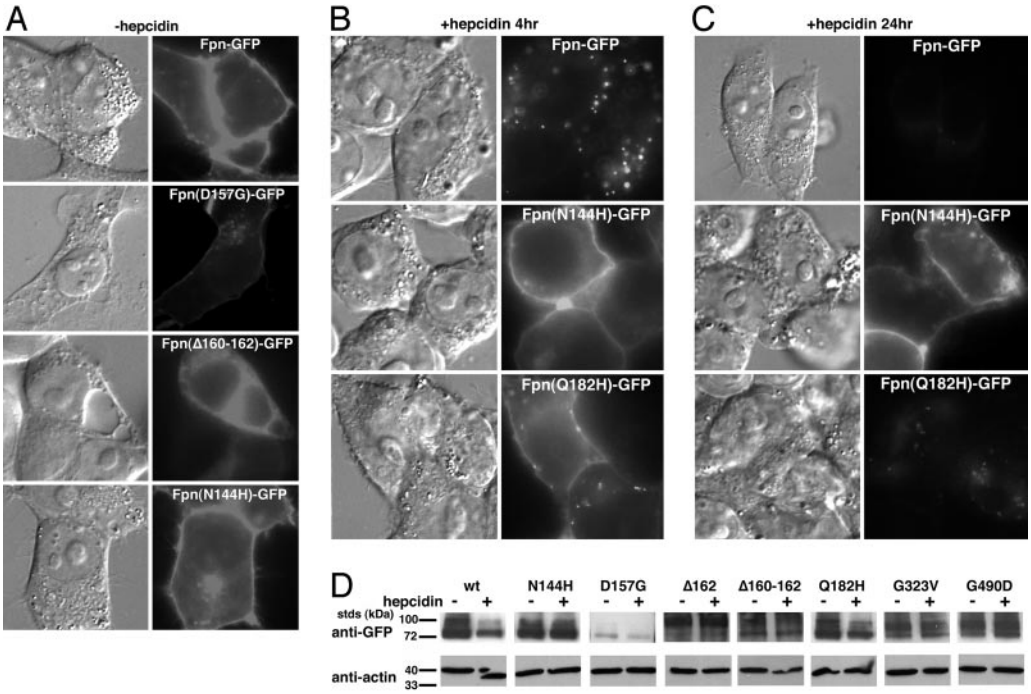

Fig. 1. Disease-inducing Fpn mutations affect localization and response to hepcidin. (*A–C*) HEK293T cells were transiently transfected with plasmids containing WT Fpn-GFP, Fpn(D157G)-GFP, Fpn(Δ160-162)-GFP, or FpnN144H-GFP, and localization was assessed by epifluorescent microscopy. Eighteen to 24 h after transfection, cells were incubated with 1 μg/ml hepcidin for 4 (*B*) and 24 (*C*) h and examined for Fpn-GFP localization. (*D*) Cells were incubated with or without 1 μg/ml hepcidin for 4 h, and extracts were analyzed by Western blot analysis using antibody to GFP and an antibody to actin as a loading control.

were not expressed at the cell surface, did not show any response to hepcidin even after 24 h (data not shown). Western blot analysis showed that, with one exception, all Fpn mutants were expressed at concentrations comparable to WT Fpn (Fig. 1*D*). The only exception was mutant D157G, which is expressed at ≈10% of WT Fpn levels. Sequence analysis showed that the decrease in expression of mutant D157G could not be ascribed to incidental mutations in either the coding sequence or the promoter. We note that Fpn often appears as a double band in cells that are expressing the protein from the CMV promoter.

Western blot analysis confirmed hepcidin-induced degradation of WT Fpn. However, Fpn mutants (N144H and D157G) that showed no internalization upon exposure to hepcidin also showed no change in Fpn protein level. Fpn mutant Q182H, which showed some hepcidin-mediated internalization, also showed reduction in protein levels in response to hepcidin. As expected, Fpn mutants that showed defective cell surface localization were not affected by hepcidin. The data on cellular localization and hepcidin response for all mutants are summarized in Table 1.

Incubation of cells in high-iron medium leads to cellular uptake of iron, increased cytosolic iron, and accumulation of the iron storage protein ferritin. Expression of the iron exporter Fpn decreases cytosolic iron and ferritin levels, even in the presence of iron-containing media (8). Cells expressing Fpn mutants (Δ162, Δ160-162, G323V, and G490D) that did not properly localize to the cell surface had higher levels of ferritin than cells expressing WT Fpn (Fig. 2*A*, open bars). Ferritin levels in these cells, however, were slightly lower than those in cells transfected with a control vector expressing GFP. This result is consistent with the localization data that indicate that some of the mutant Fpn localized to the cell surface. Fpn mutants N144H and Q182H had no defect in subcellular distribution and localized correctly to the cell surface. Cells expressing these Fpn mutants had ferritin levels similar to those expressing WT Fpn, indicating no obvious defect in iron transport activity. In cells expressing Fpn mutant D157G, which had normal cell surface localization but low expression levels, ferritin levels were still reduced in comparison with control cells (cells expressing GFP) not expressing Fpn, indicating that the mutant protein exported iron.

Addition of hepcidin to cells expressing WT Fpn resulted in an increase in ferritin, reflecting the loss of the iron exporter from the surface and iron retention (8) (Fig. 2*A*, filled bars). Addition of hepcidin to cells expressing Fpn mutant Q182H also increased ferritin, showing that the Q182H protein was removed from the cell surface. The level of ferritin, however, did not reach that of WT ($P < 0.002$), suggesting a delay in hepcidin-induced degradation. The other Fpn mutants showed no change in ferritin levels upon exposure to hepcidin. Importantly, Fpn mutant N144H, which showed a normal localization pattern, continued to export iron even in the presence of hepcidin. Similarly, in cells transfected with the Fpn mutant D157G whose protein product was expressed at lower levels than either WT or other Fpn mutants, ferritin levels were unaffected by the presence of hepcidin.

The decreased response of Fpn mutants to hepcidin could reflect impaired hepcidin binding or an altered response subsequent to hepcidin binding. To distinguish between these possibilities, we assayed the binding of ^{125}I-hepcidin to cells expressing WT or Fpn mutants (Fig. 2*B*). Mutants with the localization defect (Δ162, Δ160-162, G323V, and G490D) did not significantly bind ^{125}I-hepcidin. Mutants with normal localization (N144H and Q182H) bound ^{125}I-hepcidin similar to WT Fpn. No binding of ^{125}I-hepcidin was seen in cells expressing Fpn mutant D157G, but we suspect that the expression levels were too low to detect binding, suggesting that defective internalization of properly localized Fpn mutants does not result from an inability to bind hepcidin but from an inability to respond to bound hepcidin. In addition, binding of hepcidin did not diminish the ability of Fpn to transport iron, suggesting that Fpn internalization is necessary to decrease iron export.

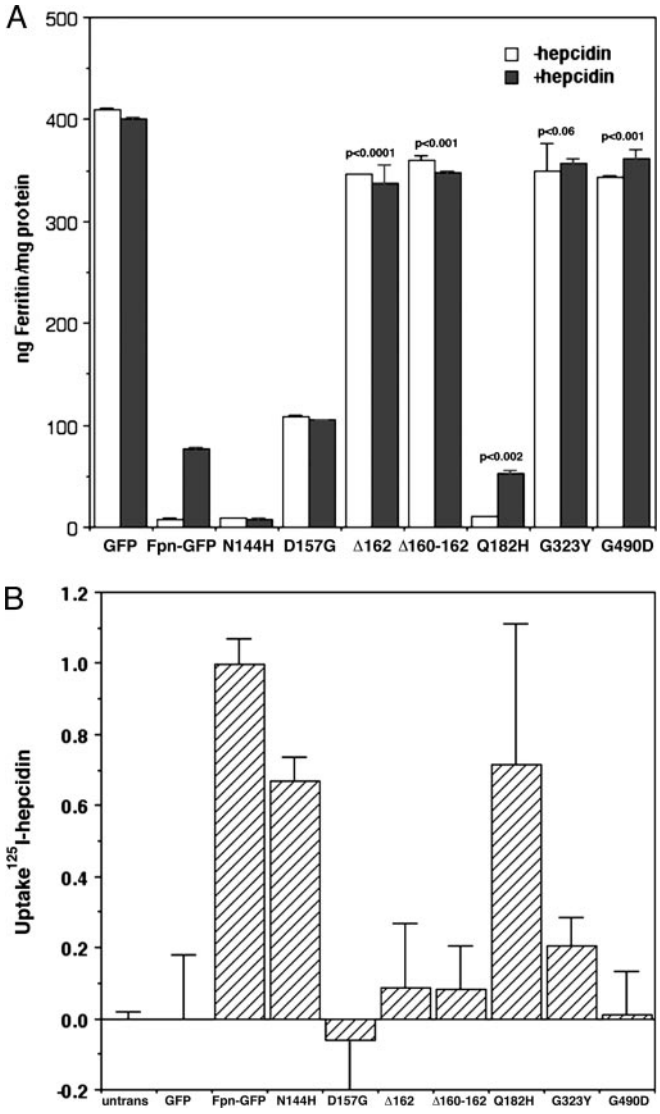

Fig. 2. Fpn mutations affect intracellular ferritin levels. (*A*) HEK293T cells were transiently transfected with plasmids containing WT Fpn-GFP or mutant Fpn-GFP. Eighteen hours after transfection, cells were cultured with ferric ammonium citrate (FAC) (20 μM iron). After FAC loading (24 h), cells were incubated with 100 μM cycloheximide (1 h) followed by 1.0 μg/ml hepcidin (4 h), and ferritin levels were determined by ELISA. Error bars are the standard deviation of three experiments. *P* values were calculated by using a Student *t* test. (*B*) ^{125}I-hepcidin was added to HEK293T cells expressing WT Fpn-GFP or mutant Fpn-GFP, and cell-associated radioactivity was measured. Each bar represents the average of four to six measurements. The data were normalized to the amount of radioactivity bound to WT Fpn-expressing cells, and the amount of radioactivity bound to untransfected cells was subtracted as background for each point. The relative values were further normalized for the amount of Fpn-GFP expressed in each sample as determined by Western blotting.

Fpn Is Multimeric. Hemochromatosis due to mutations in Fpn is inherited as a dominant disorder. All identified Fpn mutants are missense mutants: No nonsense mutations have been identified to date. The dominant inheritance of the disorder could be explained if the functional unit of Fpn was multimeric, with the mutant Fpn acting as a dominant negative by associating with the WT Fpn. To test the possibility that Fpn is multimeric, we coexpressed two different epitope-tagged Fpns in the same cell (FLAG and GFP) and tested for coimmunoprecipitation. Immunoprecipitates of Fpn-FLAG contained Fpn-GFP but not plasma membrane proteins such as EGF receptor (Fig. 3*A*). Similarly, when anti-GFP was used

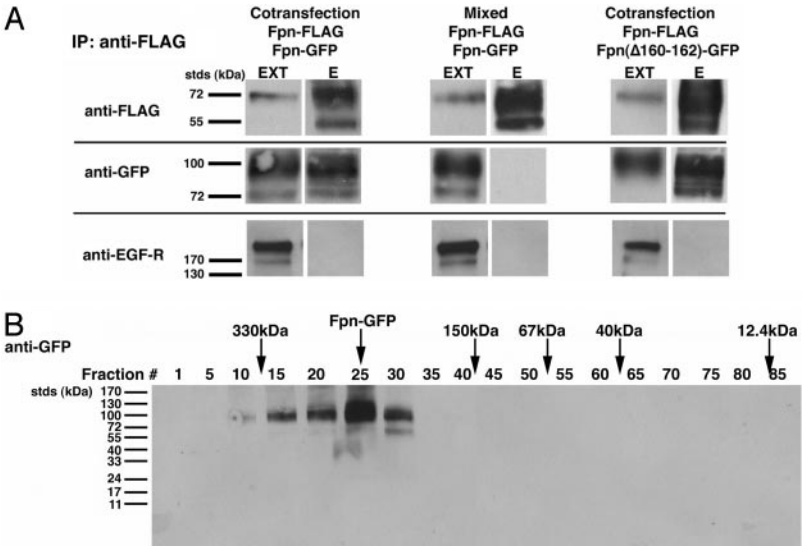

Fig. 3. Immunoprecipitation of Fpn demonstrates that Fpn is a multimer. (*A*) (*Left*) HEK293T cells were transiently transfected with plasmids containing WT Fpn-FLAG and Fpn-GFP. Cell extracts (1.5 mg of protein equivalent to one confluent 6.0-cm plate) were obtained after 24 h and immunoprecipitated by using anti-FLAG. EXT, cell extracts before immunoprecipitation; E, FLAG-specific elutions. (*Center*) Extracts were prepared from cells expressing either Fpn-FLAG or Fpn-GFP. The extracts were mixed and then subjected to immunoprecipitation. (*Right*) HEK293T cells were transfected with plasmids containing WT Fpn-FLAG and Fpn(Δ160-162)-GFP, and cell extracts were immunoprecipitated by using anti-FLAG. Western blots were analyzed for Fpn-FLAG, Fpn-GFP, and EGF receptor. (*B*) HEK293T Fpn cells were induced to express Fpn-GFP by the addition of ponasterone for 24 h. Cells were solubilized by the addition of 1.0% Triton X-100. Samples were applied to a Superdex 200 FPLC column that had been equilibrated with Triton X-100. Fractions were collected and analyzed for Fpn-GFP by Western blot analysis.

for immunoprecipitation, Fpn-FLAG was detected in immunoprecipitates (data not shown). No coimmunoprecipitation was detected when extracts of cells expressing only Fpn-FLAG were mixed with extracts of cells expressing only Fpn-GFP before immunoprecipitation. Importantly, coexpression of mutant Fpn(Δ160-162)-GFP, mutant Δ162, or G323V (data not shown) with WT Fpn-FLAG showed that mutant Fpn also associated with the normal protein (Fig. 3*A*). These results suggest that Fpn is multimeric. To determine the size of the Fpn multimer, detergent extracts of cells expressing Fpn-GFP were analyzed by size-exclusion chromatography. GFP-tagged Fpn (97 kDa) eluted at a mass much larger than a monomer (Fig. 3*B*). Similar results were obtained with extracts of cells expressing Fpn-FLAG, a much smaller epitope (data not shown). The apparent mass of Fpn-GFP (235 kDa) suggests that it might be dimeric or trimeric.

If Fpn is a multimer, then expression of a mutant protein with a defect in plasma membrane localization could affect the subcellular distribution of WT Fpn. Expression of two different epitope-tagged WT Fpns in the same cell did not affect the cell surface localization of either Fpn (Fig. 4*A*). Expression of Fpn(Δ160-162)-GFP, which shows no cell surface localization (see Fig. 1), affected the surface distribution of WT Fpn-FLAG. Although some WT Fpn-FLAG was found at the cell surface (Fig. 4*A*, arrows), a significant amount was found within the cell (Fig. 4*A*, arrowheads). Interestingly, some mutant Fpn-GFP was now found at the cell surface. WT Fpn localization was also altered when WT Fpn was coexpressed with Fpn mutant Δ162 or G323V (data not shown). This effect of Fpn mutants on WT localization is specific for Fpn. Expression of Fpn mutants did not affect the endosomal localization of the Tf-R (Fig. 4*A*). The effects of the mutant Fpn on the distribution of WT Fpn lasted throughout the transfection period (18–48 h).

Coexpression of WT Fpn with mutants defective in hepcidin-mediated internalization (N144H) decreased the hepcidin-mediated internalization of WT Fpn (Fig. 4*B*, arrows). Lack of Fpn internalization would result in continuous iron export, and, as predicted, coexpression of Fpn (N144H)-GFP with WT Fpn-FLAG resulted in low ferritin levels even in the presence of hepcidin, indicating that the mutant Fpn prevented the internalization of WT Fpn-FLAG (Fig. 4*C*). Coexpression of WT and mutant Fpn (Δ160-162 and Δ162), which do not localize to the cell surface, affects the ability of WT Fpn to reduce ferritin levels. The mutant Fpn expressed by itself does not respond to hepcidin because it does not reach the cell surface. When expressed with WT, the mutant/WT complex responds to hepcidin, resulting in an increase in cellular ferritin.

Transiently expressed Fpn mutants are expressed at comparable levels, with the exception of Fpn D157G. This mutant Fpn, even when expressed by a CMV promoter, accumulates to levels that are only 10% of other Fpn. We considered that the low expression level of Fpn D157G reflects decreased protein stability or decreased translation. If Fpn D157G is made and can form a multimer with WT Fpn, then we predict that coexpression of mutant D157G with WT Fpn should lead to a reduced level of WT Fpn. Decreased levels of WT Fpn-FLAG were seen in cells transiently transfected with WT Fpn-FLAG and mutant Fpn(D157G)-GFP compared with cells expressing WT Fpn-FLAG and WT Fpn-GFP (Fig. 5). Coexpression of Fpn(D157G)-GFP and WT Fpn-FLAG resulted in decreased iron export, as shown by increased levels of ferritin compared with cells expressing WT Fpn-FLAG and Fpn-GFP. This result suggests that the low level of expression of FpnD157G results from decreased protein stability and that the unstable protein can affect the stability and activity of WT Fpn.

Discussion

Fpn disease (HH type IV) is a form of iron overload caused by mutations in the *SLC40A1* gene encoding Fpn. Unlike other types of HH, Fpn disease shows dominant genetic inheritance. The clinical presentation is heterogeneous: Some patients present with macrophage iron deposition and high ferritin levels despite normal transferrin saturation (13), whereas others develop abnormalities similar to typical hemochromatosis, such as elevated transferrin saturation and iron deposition in hepatocytes (14, 15). This study provides the molecular basis for understanding how distinct Fpn mutations contribute to development of a particular phenotype and why Fpn mutations are dominant.

Fpn mutations separated into two groups: One group manifested the loss of iron export function; the other retained full iron export activity. For Fpn mutants Δ162, Δ160-162, G323V, and G490D, the loss of cellular iron export function was due to mislocalization of the mutant protein. Although the level of expression of these Fpn mutants was comparable to the WT as determined by Western blotting, epifluorescent microscopy showed that mutant Fpn was primarily intracellular, in contrast to WT Fpn, which is almost exclusively localized to the plasma membrane. These mutants did not bind hepcidin and did not show any hepcidin-induced degradation of Fpn. The lack of membrane expression of Δ162, Δ160-162, G323V, and G490D Fpn mutants resulted in reduced cellular iron efflux, as reflected by the high ferritin levels in cells expressing these

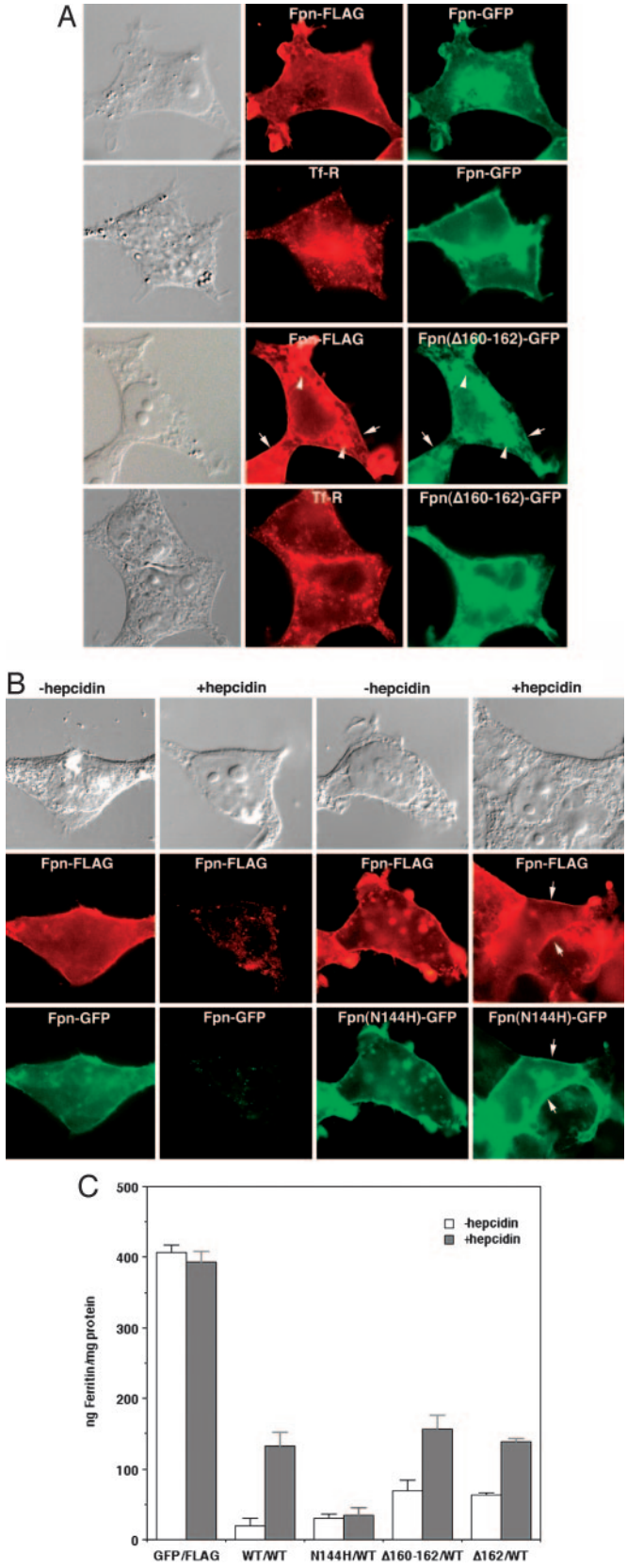

Fig. 4. Coexpression of mutant and WT Fpn alters the surface distribution of WT Fpn and affects Fpn response to hepcidin. (*A*) HEK293T cells were transiently cotransfected with plasmids containing either WT Fpn-FLAG and Fpn-GFP or Fpn-FLAG and Fpn(Δ160-162)-GFP. Localization of different Fpn as well as the Tf-R was assessed by immunofluorescence microscopy. (*B*) HEK293T cells were transiently transfected with plasmids containing WT Fpn-FLAG and Fpn-GFP or Fpn-FLAG and Fpn(N144H)-GFP. After 24 h, cells were incubated with 1 μg/ml hepcidin (4 h), and localization of Fpn-FLAG/GFP was assessed by immunofluorescence microscopy. (*C*) Cells were transfected with plasmids containing WT Fpn-FLAG and Fpn-GFP, Fpn-FLAG and Fpn(N144H)-GFP, Fpn-FLAG and Fpn(Δ160-162)-GFP, or Fpn-FLAG and Fpn(Δ162)-GFP. Eighteen hours posttransfection, cells were cultured with ferric ammonium citrate (20 μM iron). After incubation with iron for 24 h, cells were incubated with 100 μM cycloheximide (1 h) followed by 1 μg/ml hepcidin (4 h), and ferritin levels were determined by ELISA. The data are presented as mean ± standard deviation ($n = 3$).

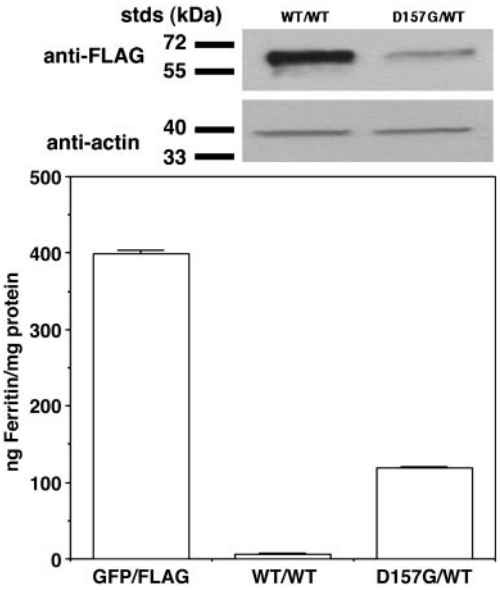

Fig. 5. Coexpression of mutant and WT Fpn affects the stability of Fpn and its ability to export iron. (*Upper*) HEK293T cells were transiently cotransfected with plasmids containing WT Fpn-FLAG, Fpn-GFP, or Fpn(D157G)-GFP. After 24 h, samples were extracted and analyzed by Western blot. The Western blots were probed with antibodies to actin as a control for loading. (*Lower*) Ferritin levels were assayed in cells transiently transfected with GFP/FLAG vector control, Fpn-FLAG and Fpn-GFP, and Fpn-FLAG and Fpn(D157G)-GFP.

mutants. The reduction in iron efflux causes a bottleneck in tissues that generate the largest iron flows (i.e., macrophages involved in the recycling of iron from senescent erythrocytes). Our *in vitro* findings are in agreement with the phenotypic characteristics of the patients with these mutations: iron accumulation in macrophages, with high ferritin levels and low to normal transferrin saturation.

To a lesser extent, the D157G mutation also affected iron efflux *in vitro*. Although the mutant correctly localized to the membrane, the level of its expression was greatly reduced in comparison with the WT, resulting in cellular iron retention. Interestingly, the addition of hepcidin did not increase iron retention, and the mutant was shown to be resistant to hepcidin-induced degradation. Although the clinical description of the patient was incomplete (16), the mutation appears to confer a phenotype similar to other mutations that mislocalize Fpn.

In contrast, mutation N144H correctly localized to the membrane and showed normal iron efflux activity. However, this mutant was resistant to regulation by hepcidin. The addition of hepcidin did not reduce cellular iron export as determined by ferritin levels, and no hepcidin-induced degradation of the mutant protein was detected by Western blotting. Because hepcidin binding is not altered, we surmise that the mutation affects the Fpn domains required for internalization and degradation. The lack of Fpn regulation by hepcidin would be

expected to mimic hepcidin deficiency and yield a phenotype similar to classical hemochromatosis, with inappropriately high duodenal absorption of iron, increased transferrin saturation, and iron deposition in hepatocytes. Of 12 reported subjects carrying the N144H mutation, 6 had elevated serum ferritin, and of those, 3 had transferrin saturations >80% (17). In addition, patients with two other mutations affecting the same residue [N144T (18) and N144D (15)] were described to have high serum ferritin and transferrin saturation and massive liver parenchymal iron accumulation with some Kupffer cell iron loading. Although other genetic and environmental variables must contribute to the phenotypic picture, it is clear that N144X mutations predispose to a distinct phenotype resembling classical hemochromatosis.

The Q182H Fpn mutation was most similar to WT; it showed normal membrane localization and hepcidin-induced degradation, although internalization by hepcidin was delayed. Despite the limited clinical information available for this mutation, surprisingly, it was associated with high ferritin levels and normal transferrin saturation, and the mechanism remains to be more fully characterized.

An overlapping set of mutations was also analyzed by Schimanski *et al.* (19), who similarly found altered subcellular localization and impaired iron export of Δ162 and G490D and normal localization and iron export function of N144H and several other mutants. More recently, Drakesmith *et al.* (20) demonstrated that some Fpn mutants, including Fpn(N144H), were partially resistant to hepcidin. We show here that Fpn mutants act as dominant negatives. Coimmunoprecipitation of Fpn with different epitope tags, as well as the detection of a complex much larger than the monomer by size-exclusion chromatography, indicated that the functional unit of Fpn is a multimer. This result is consistent with the observation by Aguirre *et al.* (21), who suggested that Fpn was dimeric. Coimmunoprecipitation showed that Fpn mutants could also form multimeric complexes with WT Fpn. Coexpression of the WT Fpn with mutants defective in cell surface localization led to mislocalization of the WT Fpn. The altered location of Fpn had functional consequences; measurement of intracellular ferritin levels indicated that iron export was diminished. Coexpression of WT Fpn with mutants that are unresponsive to hepcidin also altered the hepcidin-mediated internalization of WT Fpn. Accordingly, the regulation of iron export was affected: Addition of hepcidin failed to cause cellular iron retention, indicating that WT/mutant complexes were permanently turned on. Expression of Fpn(D157G), which when expressed by itself accumulates to lower levels than WT, affects the level of WT Fpn, resulting in iron retention.

Multimerization of Fpn mutants with WT Fpn suggests that Fpn disease pathogenesis conforms to the dominant-negative model. Mutations such as Δ162, Δ160-162, G323V, and G490D, which result in retention of the mutant inside the cell, alter the localization of WT Fpn. Even though some WT Fpns (i.e., a homomultimer) will be present on the cell surface, the overall iron efflux will be reduced. Although this level of Fpn may be enough for transport of iron out of the intestinal duodenum (≈1–2 mg/day), the efflux rate in macrophages recycling erythroid iron is much greater (≈20 mg/day), and decreased cell surface localization of WT Fpn will result in macrophage iron accumulation. This accumulation would lead to high ferritin levels, low transferrin saturation, and possibly borderline anemia. Eventually, anemia might increase duodenal absorption, which would progressively increase transferrin saturation. In contrast, mutations such as N144H prevent hepcidin-mediated internalization and degradation of the mutant protein and, due to multimerization, also interfere with the internalization of WT Fpn. We did not seen any effect of hepcidin addition on ferritin levels in cells expressing both WT Fpn and Fpn(N144H), but expression levels of these proteins were high, and internalization of the WT Fpn multimer from the cell surface might not be rate-limiting for iron export. As a result, iron efflux from enterocytes and macrophages is inappropriately increased, leading to increased transferrin saturation and ultimately iron deposition in hepatic parenchyma and other tissues. In contrast to our results, Schimanski *et al.* (19) did not find multimerization of Fpn mutants with WT visually, by coimmunoprecipitation, or by assessing the iron export function. They concluded that these Fpn mutations would cause disease due to haploinsufficiency. The phenotypic consequences as predicted by this model would be very similar to the dominant-negative model for mutants that show the classic disorder of Kupffer cell iron loading. The haploinsufficiency model would not explain hepatocyte iron loading. A second critical distinction between the two models arises with respect to nonsense mutations. The fact that none have been identified in patients so far would favor the multimerization model where Fpn mutants impede the function of the normal protein.

Most types of HH (due to mutations in HFE, Tf-R 2, hemojuvelin, and the hepcidin gene) are characterized by hepcidin deficiency. We provide here the molecular basis for the remaining type of hemochromatosis, Fpn disease. The disease develops due to the Fpn localization defect, resulting in a loss of iron export function, or due to resistance to negative regulation by hepcidin, resulting in a gain of iron export function. This study underscores the central role of the hepcidin–Fpn interaction in regulating iron homeostasis and in pathogenesis of iron overload diseases.

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