Role of H and L Subunits in Mouse Ferritin*

Paul Rucker‡, Frank M. Torti‡§, and Suzy V. Torti¶

From the Departments of ‡Cancer Biology, §Biochemistry, and ¶Medicine, Bowman Gray School of Medicine and the Comprehensive Cancer Center of Wake Forest University, Winston-Salem, North Carolina 27157

Ferritin is an iron-binding protein composed of two subunits, H and L. Twenty-four of these subunits assemble to form apoferritins whose subunit composition varies in a characteristic way in different tissues. Using recombinant proteins, we have assessed the role of H and L subunits in mouse ferritin function and compared these to human ferritin subunits. We report that mouse ferritin subunits exhibit considerable functional similarity to their human counterparts, including a prominent role of the H subunit in the facilitation of rapid iron uptake, and a key role of amino acid residues Glu-62 and His-65 in this process. In addition, amino acid residues important to assembly of the protein are conserved between mouse and human, permitting the formation of fully functional hybrid proteins containing both mouse and human subunits. However, murine and human ferritin H subunits also evidenced substantial functional differences; murine ferritin H showed a consistent reduction in iron uptake activity relative to human ferritin H. Creation of chimeric human/mouse ferritin H subunits by “helix swapping” mapped the domain of the protein critical to this activity difference to the DE helix. These findings suggest a novel functional role for carboxyl-terminal domains of the ferritin H subunit.

Ferritin is a protein which has as its principal function the intracellular storage of iron in a nontoxic and bioavailable form (see Refs. 1 and 2 for review). Ferritin is ubiquitously distributed in the animal and plant kingdoms and has recently been described in bacteria (3). Mammalian ferritin is composed of two subunits, termed H and L. Twenty-four of these subunits assemble to form the apoferritin protein.

In mammals, ferritin is found in most tissues. However, the composition of ferritin varies in a consistent and tissue-specific way. For example, liver contains ferritin that is predominantly of the L subunit type, whereas heart contains ferritin rich in the H subunit. This biodistribution, as well as the evolutionary conservation of a dual subunit structure, supports the hypothesis that the H and L ferritin subunits may play different and complementary roles within the protein (4). Experiments using H-rich and L-rich ferritin prepared from natural sources (5, 6), as well as recent work with recombinant proteins (7–9), have supported this concept of a functional distinction between ferritin H and L subunits and revealed a prominent role of the H subunit in rapid iron oxidation (7), and the involvement of the L subunit in protein stability and iron mineralization (8, 9).

Mouse cells and mouse models have been widely used to study iron homeostasis in health and disease (e.g. Refs. 10–14). For the most part, however, biological inferences concerning ferritin function and iron metabolism in these models have been based on analogy to human ferritin. Indeed, substantial sequence similarity exists between mouse and human ferritin subunits; mouse L and human L exhibit 82% similarity (15), whereas human and mouse ferritin H are 93% identical (16). However, the hypothesis that mouse ferritin subunits are functionally equivalent to human ferritin subunits has not been experimentally tested.

We provide here direct evidence that mouse and human ferritin subunits have similar properties, including a prominent role of the H subunit in the facilitation of rapid iron uptake, and a key role of amino acid residues Glu-62 and His-65 in this process. In addition, amino acid residues important to assembly of the protein are conserved, permitting the formation of fully functional mouse/human hybrid proteins. However, we also observed a significant functional difference between mouse and human ferritin H subunits, which we have mapped to a region of the protein not previously implicated in iron uptake activity. These findings may suggest a role of previously unsuspected domains of ferritin H in ferritin function.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Monocistronic expression plasmids for mouse ferritin L, mouse ferritin H and human ferritin L were constructed by using polymerase chain reaction to modify the coding region of pMLF27 (gift of C. Beaumont, Paris, France), MFH (16), and pLF108 (gift of J. Drysdale, Tufts University), respectively, to contain an NdeI restriction site at the initiator methionine and a BamHI site at the stop codon. These fragments were subcloned into the pET21C vector (Novagen) digested with NdeI and BamHI. The human ferritin H chain coding region was modified similarly following reverse transcription of human fibroblast mRNA from MRC-5 cells (gift of Dr. Helen Blau, Stanford University School of Medicine, Stanford, CA) with murine Moloney leukemia virus reverse transcriptase. pACYC-based expression plasmids were constructed by isolating the entire expression cassette of the four pET21 constructs using BspEI digestion, T4 DNA polymerase fill-in, and BglII digestion followed by ligation into the pACYC177 vector (New England Biolabs, Inc.) digested with BamHI and SacI. Simultaneous expression of two subunits was achieved either by subcloning a second cDNA in tandem to the first (bicistronic expression system) or by co-expressing two separate cDNAs cloned in two separate vectors (dual vector expression system). The mouse ferritin H double point mutant containing Glu-62 → Lys and His-65 → Gly amino acid substitutions was constructed by polymerase chain reaction amplification of MFH cDNA with a 5′ primer (5′-CAAATCCTATGAGAA-3′) causing a G → A transition at amino acid residue 62 and a C → G transversion and A → G transition at amino acid residue 65. This fragment was digested with BspHI and BamHI and...

* This work was supported by Grant DK 42412 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Medical Center Blvd., Winston-Salem, NC 27157. Tel.: 910-716-9357; Fax: 910-716-7671.

¶ The abbreviations used are: MFH, murine ferritin H chain; MFL, murine ferritin L chain; HFF, human ferritin H chain; HFL, human ferritin L chain; PAGE, polyacrylamide gel electrophoresis.

† P. Rucker, F. M. Torti, and S. V. Torti, manuscript in preparation.
ligated to a BsmI, partial BspHI restriction fragment of the mouse H cDNA. The cloning junctions of all 12 constructs, 100 nucleotides surrounding the mutated region of the double point mutant, and the entirety of the HFH, HFL, MFP, and MFL coding regions were sequenced by the dideoxy chain termination method.

**Helix Swapping**—A human/mouse hybrid ferritin H subunit containing human helices A–C and mouse helices DE was constructed by digesting human ferritin H cDNA with NdeI and BsmI and ligating to the BsmI fragment of mouse ferritin H. The product was amplified using a 5′ primer containing an NdeI site and complementary to human ferritin H, and a 3′ primer containing a BamHI site and complementary to mouse ferritin H. A hybrid human A–D/mouse E subunit was constructed in the same way, except that digestion of human ferritin H cDNA was with NdeI and BstEII, and digestion of mouse ferritin cDNA was with BstEII and BamHI. A hybrid subunit containing human helices A, B, C, and E and mouse helix D was constructed by subcloning a BstEII fragment of pET21c-HFH into a BstEII-digested human A–C/mouse DE construct. All constructs were verified by restriction digestion and DNA sequencing.

**Expression and Purification of Recombinant Proteins**—All plasmid constructs were expressed in the host strain BL21/DE3 (Novagen). Ferritin was purified following induction with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma). Bacteria were pelleted; resuspended in buffer containing 10% sucrose, 20 mM Tris pH 7.5, 1 mM Pefabloc (Boehringer Mannheim), 1 mM phenylmethylsulfonylfluoride (Sigma); and sonicated. Sonication was repeated following resuspension of the pellet in the same buffer containing 0.2% Triton X-100 and 100 mM NaCl. The supernatant was heated to 70°C for 15 min, applied to a 10–40% sucrose gradient, and centrifuged at 100,000 × g at 4°C for 18 h. 0.5-mL fractions were collected, assayed for protein content using the Bradford reagent (Bio-Rad), pooled as appropriate, dialyzed, and concentrated. In some cases, ferritin was purified as described previously (17). Iron content of ferritins isolated from bacteria was low (<0.01 μg of iron/μg of protein) (18).

**Determination of Subunit Composition**—Subunit composition of multi-subunit proteins was determined by SDS-PAGE, staining with silver or Coomasie Blue, and scanning (PDI, Pharmacia Biotech Inc.), using standard curves prepared by simultaneous electrophoresis and staining of known quantities of purified ferritin protein. Subunit composition was reproducible. For example, in seven independent colonies expressing murine ferritin H and murine ferritin L from a dual vector system. However, despite the ability of recombinant ferritin H and L to form homopolymers, these are generally not seen in vivo. Rather, a restricted subset of heteropolymers is formed that is characteristic of a given tissue. We therefore wished to determine whether the difference in the activity of murine and human ferritin H would be maintained in these more biologically relevant proteins. In order to perform these experiments, we designed experimental conditions that would allow the isolation of ferritin containing two different subunits from a single bacterial cell. In one case, expression of two different ferritin subunits was obtained by using a bicistronic mRNA; in a second case, subunits of ferritin were cloned into two different expression plasmids, which were simultaneously expressed in the same bacterial host (dual vector system) (21, 22). In both cases, ferritin subunits expressed in bacteria assembled appropriately into 24 subunit proteins, as judged by their co-sedimentation on sucrose density gradients with native apoferritin (Fig. 5 and data not shown). As shown
in Fig. 2, by combining these methods, both human and murine ferritins with a broad range of subunit composition were expressed in bacteria.

The availability of correctly assembled mouse and human ferritin proteins with a range of subunit compositions enabled us to test the dependence of initial rates of iron uptake on subunit composition, as well as to assess whether the difference in the iron uptake activity of murine and human ferritin H was preserved in ferritin H/L heteropolymers. As shown in Fig. 3, the highest rate of iron uptake in both cases was observed in H subunit homopolymers, and the lowest rate in L subunit homopolymers. In both mouse and human proteins, the rate of iron uptake in heteropolymers was proportional to H subunit content. Additionally, the relative decrement in murine ferritin H activity when compared to human ferritin H activity that was evident in homopolymers was completely preserved in heteropolymers. Thus, throughout a wide range of ferritin subunit compositions, human ferritin H was consistently more active than murine ferritin H (Fig. 3).

The Functional Properties of Individual Subunits in Mouse and Human Ferritin Proteins—In order to determine whether residues important to ferritin assembly are conserved in mouse and human ferritin subunits, we prepared four interspecies ferritin heteropolymers of varying composition: 90% human ferritin H, 10% murine ferritin L; 90% murine ferritin H, 10% human ferritin L; 50% human ferritin H, 50% murine ferritin L; and 50% murine ferritin H, 50% human ferritin L. All proteins analyzed were functionally competent. As was the case for intraspecies heteropolymers, in interspecies heteropolymers, initial rates of iron uptake were proportional to H subunit composition. As shown in Fig. 3, the relative increment of murine and human subunits by comparing pairs of interspecies hybrid proteins to function in iron uptake was then assessed (Fig. 6). All proteins analyzed were functionally competent. As was the case for intraspecies heteropolymers, in interspecies heteropolymers, initial rates of iron uptake were proportional to H subunit composition. As shown in Fig. 3, the relative increment of murine and human subunits by comparing pairs of interspecies hybrid proteins to function in iron uptake was then assessed (Fig. 6). All proteins analyzed were functionally competent. As was the case for intraspecies heteropolymers, in interspecies heteropolymers, initial rates of iron uptake were proportional to H subunit composition. As shown in Fig. 3, the relative increment of murine and human subunits by comparing pairs of interspecies hybrid proteins to function in iron uptake was then assessed (Fig. 6). All proteins analyzed were functionally competent. As was the case for intraspecies heteropolymers, in interspecies heteropolymers, initial rates of iron uptake were proportional to H subunit composition.
residues previously implicated in iron uptake (19, 23) are not included in these differences. This suggested that the difference in rate of iron uptake between these two species mapped to another domain of the ferritin H protein. Studies of ferritin secondary structure have indicated that ferritin subunits comprise five helices, termed A–E, as diagrammed in Fig. 7. Inspection of the murine and human ferritin H sequences shown in Fig. 7 revealed that amino acid differences between these proteins localized primarily to the D and E helices. We therefore used helix swapping to test whether differences in this region of the protein were responsible for the difference in catalytic activity between mouse and human ferritin H. The mouse E helix was substituted for the human E helix to create a chimeric subunit containing helices A–D of human ferritin H and helix E of mouse ferritin H (Fig. 8A). As shown in Fig. 8A, this substitution did not appreciably affect the initial rate of iron uptake, and the homopolymer formed from the self-assembly of the human H/mouse E chimeric subunit exhibited a very similar rate of iron uptake to a homopolymer of the unmodified human H subunit. However, when both the murine D and E helix were swapped for the human D and E helix in the human subunit, the initial rate of iron uptake was dramatically reduced. Substitution of the murine D helix for the human D helix was sufficient to partially but not completely effect this change (Fig. 8). Thus, residues responsible for the differences between mouse and human ferritin H map to the domain contained within the D and E helices.

**DISCUSSION**

Mechanisms regulating ferritin synthesis have been the subject of intense investigation. Iron regulates ferritin via a novel post-transcriptional mechanism (reviewed in Refs. 1 and 2). In addition, it has recently become apparent that transcriptional control plays an important role in the regulation of ferritin synthesis, particularly in response to environmental cues such as cytokines (16, 24) and hormones (25–27). Interestingly, it is the ferritin H gene that is transcriptionally modulated. This differential transcriptional regulation of ferritin H in the absence of a change in ferritin L results in a change not only in the amount of ferritin, but in its subunit composition. To appreciate the biological implications of altered ferritin subunit composition, functional differences between H and L protein subunits must be understood. Although many studies of ferritin regulation have been performed in cells of murine origin, murine ferritin protein function has not been previously as-

**FIG. 5.** Density gradient analysis of interspecies ferritin heteropolymers. Top panel, horse spleen apoferritin; bottom panel, mouse/human hybrid ferritin. Horse spleen apoferritin and recombinant ferritin containing 48% mouse H chain + 52% human L chain were layered onto 10–40% sucrose gradients and centrifuged at 100,000 × g for 18 h at 4°C. Fractions were collected and assayed for protein content. Under these conditions aldolase (158 kDa) migrated at fraction 10 (left arrow) and thyroglobulin (669 kDa) at fraction 28 (right arrow). Fraction 1 represents the top of the gradient.

**FIG. 6.** Iron uptake activity of mouse/human hybrid ferritin proteins compared to mouse and human proteins. The change in absorbance at 310 nm following the addition of 100 μM ferrous ammonium sulfate to each of the indicated proteins is shown. Subunit composition of the proteins is indicated at right as percent of the total protein represented by the H subunit. At left are proteins containing the mouse ferritin H subunit and either the mouse or human ferritin L subunit; at right are proteins containing the human ferritin H subunit, and either human or mouse ferritin L. Asterisked (**) proteins are mouse/human hybrid proteins. Curves depict results of a typical experiment and represent the average of at least two consecutive assays.

**Mouse Ferritin Subunits**

![Image of Mouse Ferritin Subunits](33355)
Mouse Ferritin Subunits

Re-assembly of ferritin has been previously achieved by utilizing renaturation of ferritins treated with denaturants (17, 28), as opposed to the strategy of co-assembly of recombinant interspecies hybrid proteins. However, we observed throughout these experiments that mouse ferritin H exhibited a consistent reduction in activity relative to human ferritin H. This was observed in both homopolymers and heteropolymers. This was an unanticipated finding, based on the high degree of evolutionary conservation between mouse and human ferritin H, a conservation that includes residues involved in iron mineralization and oxidation (19, 23). These include tyrosyl residues, which have been implicated in initial events of iron oxidation (23); Glu-27, Glu-62, His-65, Glu-107, and Gln-141, which function as metal ligands in the ferroxidase center; as well as residues 61, 64, and 67, which have been reported to contribute to iron nucleation (20, 29).

To identify domains responsible for the difference between the H subunits of human and mouse, we constructed a number of chimeric subunits in which helices of the mouse ferritin H subunit were swapped for their human counterparts. We observed that substitution of the E helix alone did not modulate the catalytic activity of the human ferritin H subunit. Since substitution of the E helix alone did not modulate the catalytic activity of the human ferritin H subunit, the three amino acid changes at the D helix (His-136 → Tyr, Asn-139 → Ser, and Ala-144 → Ser) may largely account for the difference in catalytic activity between mouse and human ferritin H. However, substitution of the mouse D helix for the human D helix did not yield a protein of complete equivalence to the mouse ferritin H subunit (Fig. 8), suggesting that alterations in the E helix may play a contributory role.

Fig. 7. Amino acid sequence comparison of mouse and human ferritin H. Domains included in helices A–E of mouse and human ferritin H are shown; the double point mutation introduced into murine ferritin H is boxed.

Fig. 8. Effect of helix swapping on iron uptake activity. A, rates of iron uptake in proteins composed of 100% human ferritin H, 100% mouse ferritin H, 100% chimeric subunit containing helices A–C of human ferritin H and helices DE of murine ferritin H (HH/mDE), 100% chimeric subunit containing helices A–D of human ferritin H and helix E of murine ferritin H (HH/mE), and 100% chimeric subunit containing helices A, B, C, and E of human ferritin H and helix D of mouse ferritin H (HH/mD). Rates were measured over the first minute in seven independent experiments; standard errors are shown. B, diagram of human/mouse chimeric subunits created by helix swapping.
the D helix have previously been reported (30). However, an alteration in residues that line the hydrophilic channel is unlikely to explain our results, since residues that line these channels, including Asp-131 and Glu-134, are conserved in mouse and human ferritin H. An alternative possibility is that conformational differences underlie the differences in catalytic activity between mouse and human ferritin H. For example, mutations in the loop that connects the D and E helix have been reported to alter the conformation of the ferritin protein (31), an effect that might in turn influence its iron uptake properties. However, we observed no gross differences in size and shape of mouse and human ferritin H homopolymers by either sucrose density gradient centrifugation (Fig. 5) or electron microscopy. In addition, those residues of the loop reported to be most crucial to maintenance of correct conformation (159–161; Ref. 31) do not differ in mouse and human ferritin H. Although we cannot exclude the existence of small but important conformational differences between mouse and human ferritin H, large conformational differences are unlikely to underlie the differences in catalytic activity between mouse and human ferritin H reported here. Further experiments, including the use of point mutants, will be required to precisely define the contribution of specific amino acid residues in the D and E helix to the function of the ferritin H subunit.

Acknowledgments—We thank Yiwen Zhao and Rong Ma for excellent technical assistance, and Barbara Morris at Novagen Laboratories for suggestions and advice. Oligonucleotide synthesis was performed in the DNA Synthesis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University, and electron microscopy was performed in the Micromed Facility of the Comprehensive Cancer Center of Wake Forest University, supported in part by National Institutes of Health Grant CA-12197.

REFERENCES

1. Theil, E. C. (1987) Annu. Rev. Biochem. 56, 289–315
2. Klausner, R. D., Rouault, T. A. & Harford, J. B. (1993) Cell 72, 19–28
3. Hudson, A. J., Andrews, S. C., Hawkins, C., Williams, J. M., Izuhara, M., Meldrum, F. C., Mann, S., Harrison, P. M. & Guest, J. R. (1993) Eur. J. Biochem. 218, 985–995
4. Levi, S., Yewdall, S. J., Harrison, P. M., Santambrogio, P., Cozzi, A., Rovida, E., Spada, S., Albertini, A. & Arosio, P. (1994) J. Mol. Biol. 238, 649–654
5. Tsuji, Y., Akebi, N., Lam, T. K., Nakabeppu, Y., Torti, S. V. & Torti, F. M. (1995) Mol. Cell. Biol. 15, 5152–5164
6. Liu, P., Henkelman, M., Joshi, J., Hardy, P., Butany, J., Iwanochko, M., Clauser, M., Dhar, M., Mai, D., Waien, S. & Olivieri, N. (1996) Can. J. Cardiol. 12, 155–164
7. Picard, V., Renaudie, F., Porcher, C., Hentze, M. W., Grandchamp, B. & Beaumont, C. (1996) Blood 87, 2057–2064
8. Mulvey, M. R., Kuhn, L. C. & Scraba, D. G. (1996) J. Biol. Chem. 271, 9851–9857
9. Tartof, C., Raveau, M. & Drapier, J.-C. (1996) J. Biol. Chem. 271, 2300–2306
10. Beaumont, C., Dugast, I., Renaudie, F., Souroujon, M. & Grandchamp, B. (1999) J. Biol. Chem. 274, 7498–7504
11. Tartof, S. V., Kwak, E. L., Miller, S. C., Miller, L. L., Ringold, G. M., Myambo, K. B., Young, A. P. & Torti, F. M. (1988) J. Biol. Chem. 263, 12638–12644
12. Santambrogio, P., Levi, S., Cozzi, A., Rovida, E., Albertini, A. & Arosio, P. (1993) J. Biol. Chem. 268, 12744–12748
13. Fish, W. W. (1988) Methods Enzymol. 158, 357–364
14. Sun, S., Arosio, P., Levi, S. & Chasteen, N. D. (1993) Biochemistry 32, 9362–9369
15. Lawson, D. M., Treffy, A., Artyumiuk, P. J., Harrison, P. M., Yewdall, S. J., Luzzago, A., Cesareni, G., Levi, S. & Arosio, P. (1989) FEBS Lett. 254, 297–310
16. Levi, S., Cesareni, G., Arosio, P., Lorenzetti, R., Soria, M., Sollazzo, M., Albertini, A. & Cortese, R. (1987) Gene (Amst.) 51, 269–274
17. Levi, S., Salfeld, J., Franceschinielli, F., Cozzi, A., Donner, M. H. & Arosio, P. (1989) Biochemistry 28, 5179–5184
18. Waldo, G. S., Ling, J., Sanders-Leehr, J. & Theil, E. C. (1993) Science 259, 796–798
19. Wei, Y., Miller, S. C., Tsuji, Y., Torti, S. V. & Torti, F. M. (1990) Biochem. Biophys. Res. Commun. 169, 289–296
20. Beaumont, C., Dugast, I., Renaudie, F., Souroujon, M. & Grandchamp, B. (1989) J. Biol. Chem. 264, 7498–7504
21. Chou, C.-C., Gatti, R. A., Concanon, P., Wong, A., Chada, S., Davis, R. C. & Salse, W. A. (1986) Mol. Cell. Biol. 6, 566–573
22. Cozzi, A., Arosio, P., Treffy, A., Harrison, P. M. & Mann, S. (1991) J. Mol. Biol. 221, 1443–1452
23. Tenfry, A., Harrison, P. M., Luzzago, A., Cesareni, G. (1989) FEBS Lett. 247, 263–272
24. Cesareni, G., Levi, S., Luzzago, A., Tatsas, P., Pernice, I. & Cesareni, G. (1992) J. Mol. Biol. 227, 532–543
25. Giulian, G. G., Moss, R. L. & Greaser, M. (1983) Anal. Biochem. 129, 277–287
26. Laemmli, U. K. (1970) Nature 227, 680–685

3 P. Rucker, W. G. Jerome, F. M. Torti, and S. V. Torti, unpublished observations.