MUTANT FERRITIN L-CHAINS THAT CAUSE NEURODEGENERATION ACT IN A DOMINANT NEGATIVE MANNER TO REDUCE FERRITIN IRON INCORPORATION

Sara Luscieti*, Paolo Santambrogio*, Béatrice Langlois d’Estaintot, Thierry Granier, Anna Cozzi, Maura Poli, Bernard Gallois, Dario Finazzi, Angela Cattaneo, Sonia Levi, Paolo Arosio

From: Dipartimento Materno Infantile e Tecnologie Biomediche Università di Brescia, viale Europa 11, 25123 Brescia, Italy, Division of Neuroscience and Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milano, Italy, Chimie et Biologie des Membranes et des Nanoobjets, UMR CNRS 5248, Bât. B8, Avenue des Facultés, Université Bordeaux 1, 33405 Talence Cedex, France, Terzo Laboratorio Analisi Chimico Cliniche, A.O. Spedali Civili di Brescia, Vita-Salute San Raffaele University, Milano, Italy.

Running head: Ferritin heteropolymers with neuroferritinopathy mutants
*The first two authors equally contributed to the work.

Address correspondence to: Prof. Paolo Arosio, Dipartimento Materno Infantile e Tecnologie Biomediche, Università di Brescia, Viale Europa 11, 25123 Brescia, Italy, Tel. +39 030 394386, Fax. +39 030 307251, email: arosio@med.unibs.it

Nucleotide insertions that modify the C-terminus of ferritin light chain (FTL) cause movement disorders named neuroferritinopathies, that are inherited with dominant transmission. The disorders are characterized by neurodegeneration and abnormal brain iron accumulation. Here we describe the biochemical and crystallographic characterization of the pathogenic FTL mutant p.Phe167SerfsX26 showing that it is a functional ferritin with an altered conformation of the C-terminus. Moreover we analyze functional and stability properties of ferritin heteropolymers made of 20-23 H-chains and 1-4 L-chains with representative pathogenic mutations or truncated of the last 10-28 residues. All the heteropolymers containing the pathogenic or truncated mutants had a strongly reduced capacity to incorporate iron, both when expressed in E. coli, and in vitro when iron was supplied as Fe(III) in the presence of ascorbate. The mutations reduced also the physical stability of the heteropolymers. The data indicate that even few mutated L-chains are sufficient to alter the permeability of 1-2 of the 6 hydrophobic channels and modify ferritin capacity to incorporate iron. The dominant negative action of the mutations explains the dominant transmission of the disorder. The data support the hypothesis that neuroferritinopathies are due to alterations of ferritin functionality and provide new input on the mechanism of the function of isoforms.

Ferritins are ubiquitous iron storage molecules with a major role in the control of cellular iron availability. Cytosolic ferritins are heteropolymers composed by tissue-specific proportions of H- and L-chains, while mitochondrial ferritins are homopolymers (1). They sequester and incorporate iron in their large cavity by complex reactions that involve Fe(II) oxidation at the ferroxidase site of H-chains, iron hydrolysis and mineralization, which is facilitated by acidic residues of L-chains (2). The cellular capacity of sequestering iron is mainly related to the level of the H-chain (3), which regulates the size of cellular labile iron pool, potentially toxic for the production of reactive free radical species. Deletion of H-ferritin in mouse models is embryonically lethal (4), while its conditioned deletion after birth strongly increases iron toxicity and oxidative damage (5). Pathogenic mutations of the H-chain have not been reported yet, and all the ferritin genetic disorders so far identified interest L-chain and have dominant transmission. The most common one is associated with mutations of the Iron Responsive Element (IRE) in the 5'UTR of the transcript, which reduce or abolish its iron-mediated suppression, resulting in a constitutive L-ferritin upregulation (6,7). This disorder, named Hereditary Hypoferritinopathy with cataract syndrome (OMIM code #600886) is characterized by high levels of serum and tissue L-ferritins and by early onset bilateral cataract, caused by the formation of L-ferritin microcrystallines in the lens (8). More severe and
rare is the group of diseases named neuroferritinopathies (9) or hereditary ferritinopathies (10) (OMIM \#606159). Clinically these disorders are characterized by abnormal involuntary movements and cognitive decline which often appear in the 3rd to 6th decade of life. The neuropathology of the disorders is characterized by iron accumulation in the basal ganglia of the brain detectable by MR imaging (11) and by intracellular ferritin inclusion bodies in the glia and neurons of central nervous systems and other organs.

Six pathogenic mutations have been identified so far (9,10,12-16). They are all private mutations found in single families, except the 460InsA found in several patients of North Anglia. These mutations consist in insertions or duplications that introduce frameshifts with fully penetrant effect, the exception being the missense Ala96 → Thr. The insertions make the subunit longer than the native one: one nucleotide, as 460InsA, introduces an extension at the C-terminus of four amino acids, and two nucleotides, as 498InsTC, introduce a 16-amino acid extension. The insertions-duplications modify the L-chain: one nucleotide, as 498InsTC, introduces a 16-amino acid extension. The insertions-duplications modify the L-chain starting from residue 148, 154 and 167 of the protein made of 175 amino acids, and are predicted to alter the protein from the last two turns of the D-helix to the first half of E-helix (Fig. 1). Most previous mutational studies on ferritin were performed on the H-chain, which is structurally similar to the L-one. They showed that the extension of few residues at the C-terminus or the fusion to a short peptide, has no major effects on ferritin stability or functionality (17-20). The truncation at the residue equivalent to 158 of L-chain produced a ferritin that could not retain an iron core (3), while the deletion of the last 28 residue (equivalent to 24 residues of the L-chain) inhibited ferritin assembly when expressed in E. coli (21). More recently it was shown that nucleotide insertions in the sites equivalent to those of the L-chain progressively decreased H-ferritin solubility and functionality with increasing the length of the substitution (18).

The two pathogenic L-ferritin (FTL) mutants corresponding to FTL460InsA and to FTL498InsTC (here named L154fs and L167fs) were expressed in E. coli and characterized (22,23). The mutant L154fs was shown to be poorly soluble, to coassemble with H- and L-ferritins, and to reduce the functionality of the ferritin heteropolymers (23). In contrast, the mutant L167fs was shown to be as soluble as L-wild type (Lwt), to be less thermostable and to form aggregates when incubated with an excess of Fe(II) in aerobic conditions (22,24,25). It was proposed that these iron-rich ferritin aggregates contribute to the formation of the ferritin bodies in vivo and to alter ferritin functionality (26,27). Studies on cellular models showed that the expression of L154fs and of L167fs caused remarkably similar phenotypes, with the accumulation of endogenous ferritins, an increase of labile iron pool and of oxidative damage, joined to a reduction of ferritin half life and proteasome activity (22). This indicated that the two mutants acted in a similar way to inhibit the activity of isoferritins, and that the formation of ferritin aggregates is not a primary cause of the phenotype. The common property of the two mutants is a major conformational alteration of the C-terminus, which was exposed to solvent and accessible to proteolytic enzymes. Moreover, the expression of human L167fs in transgenic mice was shown to cause a phenotype with cytosolic and intranuclear ferritin aggregates analogous to those found in the patients (27), and to cause a deregulation of brain iron with upregulation of the endogenous ferritin, downregulation of transferrin receptor and reduced RNA binding activity of IRP1 (26).

Altogether these data do not establish if the primary cause and dominant transmission of the disorders is linked to the acquisition of a new function of the mutants, such as a decreased solubility and aggregate formation, or if is due to a dominant negative alteration of ferritin functionality. In this work we analyzed the structural, crystallographic and functional properties of recombinant L167fs expressed in E. coli. They resulted similar to those of FTL, except for a disordered conformation of the C-terminus around the 4-fold axis. We also produced ferritin heteropolymers made of 20-23 H-chains and 1-4 L-mutant chains. The ones containing the pathogenic mutants caused by nucleotide insertions as well as those truncated at the first residue of the frameshift incorporated less iron than the ones containing Lwt type, under conditions that mimic the physiological ones. In contrast the one containing the A96T point mutations behaved like Lwt. The data demonstrate that modifications of the C-terminus act in a dominant negative manner probably by altering the permeability of the ferritin shell. The results support the hypothesis that hereditary ferritinopathies are caused by alterations of ferritin functionality and provide new data on the mechanism of isoferritin functionality.
**EXPERIMENTAL PROCEDURES.**

*Plasmid construction.* Cloning of human L-ferritin cDNA was described in ref (27). To generate the L167fs mutant we cloned the cDNA of Lwt with a portion of the 3'UTR and then inserted a thymine and a cytosine in the proper position by oligonucleotide-directed mutagenesis. The construct was subcloned into the pDS20pTrp vector to obtain the pDS-L167fs plasmid for expression in *E. coli*. The fragment for the H-chain was amplified by PCR from plasmid pUD-Hwt (28) and subcloned into pET12b vector without OmpT (Novagen) digested with *BamHI* (pET-Hwt). Polyclonotypic vector was constructed by subcloning the L-ferritin cDNA downstream the cDNA of H-ferritin into the pET-Hwt vector. The cDNA for the Lwt was amplified by PCR from plasmid pDS-Lwt (27) inserting restriction sites *BamHI* at the termini and the Shine-Delgarno sequence (AAGGAG) upstream the ATG. The fragment was subcloned into pET-Hwt digested with *BamHI* (pET-H/Lwt). For the expression of the heteropolymers with the L-ferritin mutants the cDNA of L154fs and L167fs were subcloned in place of Lwt to obtain the pET-H/L154fs and pET-H/L167fs. The vector pET-H/L154fs was then subjected to site-directed mutagenesis to produce the pET-H/L148fs. The vector pETHLwt was subjected to site directed mutagenesis to introduce the A96T mutation and the stop codons in position 148, 154 and 167.

*Recombinant proteins.* Recombinant H- and L-chain ferritins were expressed and purified as before (29). The same procedure was used for L167fs mutant; the proteins were maintained in buffer containing anti-protease agents (1 mM benzamidine, 1 mM PMSF, 0.1 mM aprotinin, 10 µM leupeptin, 1 mM pepstatin) to avoid degradation. The purity of ferritins was judged by analysis of protein on 15% acrylamide SDS-PAGE stained with Coomassie blue. Iron was removed by incubation with 1% thioglycolic acid, pH 5.5, and 2,2-bipyridine followed by dialysis against 0.1 M Hepes buffer, pH 7.0. Protein concentration was determined with BCA reagent (Pierce, Cheshire, UK) calibrated on bovine serum albumin. Purified ferritin homopolymers were denatured in 0.1 M phosphate, pH 3.0, 6 M guanidine-HCl at 4°C for 16 hours. The H- and L-chains heteropolymers were obtained in *vitro* by mixing the two denatured ferritin chains in different molar ratios and allowed to renature, after at least ten fold dilution in 0.1 M phosphate buffer, pH 7.4, 1 mM DTT, for two hours at room temperature (30). To study iron incorporation of the heteropolymers during expression in *E. coli*, the cells transformed with the bicistronic vectors were grown in M9 minimal growth medium until they reached 0.6 OD and then induced for 3h with 0.4 mM IPTG. The cells were harvested, homogenized and the soluble supernatant heated at 65°C for 10 min. The ferritins were then analysed by nondenaturing PAGE. For stability and ferroxidase activity studies, the heteropolymers were purified by gel filtration on Sepharose 6B columns, to obtain preparation that were >95% pure, as judged by SDS-PAGE. Dynamic Light Scattering or Photon Correlation Spectroscopy measurements were performed using DynaPROMS/X on ferritins concentrated up to 10 mg/ml.

*NanoSpray-ESI-MS analysis of intact proteins.* Protein sample solution was acidified to 10% of formic acid for direct nano-ESI-MS analysis on an API QStar PULSAR (PE-Sciex Instruments, Canada) mass spectrometer equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). Analysis was performed in positive ion mode and the HV potential was set-up around 800 V. Full scan mass spectra ranging from m/z 600 to 2200 Da were collected. Peak deconvolution with the Bayesian Protein Reconstruct was done using BioAnalyseSTM software 1.1.5 for molecular mass determination.

*MALDI-TOF MS analysis of digested proteins.* Bands of interest were excised from gels, subjected to reduction by 10 mM DTT, alkylation by 55 mM IAA, and finally digested with trypsin overnight (Roche) (31). One µL aliquots of the supernatant were used for MS analysis on a MALDI-TOF Voyager-DE STR (Applied Biosystems) mass spectrometer using the dried droplet technique and alpha-cyano-4-hydroxycinnamic acid as matrix. Peak list was obtained by peak de-isotoping. Spectra were accumulated over a mass range of 750–4000 Da with a mean resolution of about 15000. Spectra were internally calibrated using matrix signals and trypsin autolysis peaks then processed via Data Explorer software version 4.0.0.0 (Applied Biosystems).

**Ferritin stability.** Denaturation of ferritin was evaluated on samples (50 µg/ml) following their incubation for 18 hours at 4°C in 0.1 M phosphate buffer, pH 7.4, 1 mM DTT, with various guanidine-HCl concentrations. Conformational state was estimated from fluorescence spectra (excitation at 295 nm), using, as standards, ferritin either in 0.1 M phosphate, pH 7.4, (native state) or in 0.1 M phosphate, pH 3.0, 6 M guanidine-HCl (denatured state). Fluorescence spectra were measured with a Kontron SFM25.
The concentration of about 5 and H-ferritin, respectively. They were used at a LF03 and rH02 were used for the staining of L-nondenaturing PAGE the monoclonal antibodies apoferritins 0.1 μM in 0.1 M Hepes pH 6.5 were supplemented with 0.1 mM freshly made ferrous ammonium sulfate, and the development of amber color of Fe(III) was followed at 310 nm. In other experiments, 0.2 μM ferritins in 0.2 M acetate buffer pH 6.0 containing 4 mg/ml human apotransferrin were supplemented with 1 mM freshly made ferrous ammonium sulfate and the development of pink Fe(III)-transferrin complex was followed at 460 nm for 5 min.

**Iron incorporation in vitro.** Apoferritins (1 μM, 0.5 mg/ml) were incubated for two hours at room temperature with 0.5-4.0 mM freshly made ferrous ammonium sulfate in 0.1 M Hepes buffer, pH 7.0. The samples were run on non-denaturing 7% polyacrylamide gels and stained for protein (Coomassie blue) or for iron (Prussian blue). In other experiments, apoferritins (0.2 μM, 0.1 mg/ml) were incubated with 0.1 mM 55Fe (ferric citrate) and 1 mM ascorbic acid in 0.1 M Hepes buffer, pH 7.0 for two hours at room temperature. The samples were run on non-denaturing 7% polyacrylamide gels, dried and the presence of 55Fe revealed by autoradiography. To study in vitro iron incorporation in the heteropolymers produced by the bicistronic vectors, equal amounts of the ferritins were separated on nondenaturing PAGE and then treated for iron removal by 2 h incubation with 1% thioglycolic acid, pH 5.5, and 2,2-bipyridine. The gels were extensively washed with 0.1 M Hepes buffer, pH 6.5 and used to study ferritin iron uptake. In some experiments the gels were incubated in 200 μM ferrous ammonium sulfate for 15 min in Hepes buffer, washed and stained with Prussian blue. Alternatively the gels were incubated in 20 mM Tris-HCl pH 7.0 with 1 mM ferric ammonium citrate and 1 mM Na ascorbate for 15 min, washed and then stained with Prussian blue. For enhancing after Prussian blue staining, the gels were washed and incubated with 0.05% dianisomobenzidine and 0.05% H2O2 for 15 min.

**Immunological methods.** In western blotting of nondenaturing PAGE the monoclonal antibodies LF03 and rH02 were used for the staining of L- and H-ferritin, respectively. They were used at a concentration of about 5 μg/ml. For SDS-PAGE we used the polyclonal rabbit anti ferritin antibody (Sigma) that preferentially recognizes the L-chains. Antibody binding was revealed using HRP labeled secondary antibody and ECL detection.

**Ferroxidase activity.** To study the ferroxidase activity of the ferritins and heteropolymers the apoferritins 0.1 μM in 0.1 M Hepes pH 6.5 were supplemented with 0.1 mM freshly made ferrous ammonium sulfate, and the development of amber color of Fe(III) was followed at 310 nm. In other experiments, 0.2 μM ferritins in 0.2 M acetate buffer pH 6.0 containing 4 mg/ml human apotransferrin were supplemented with 1 mM freshly made ferrous ammonium sulfate and the development of pink Fe(III)-transferrin complex was followed at 460 nm for 5 min.

**Crystallization and structure determination of L167f.** The first crystallization assays of L167fs were performed with a Cartesian Honeybee 961 robot (Genomic Solutions) using Greiner 96 well sitting drop crystallization plates. Different commercial screens were tested (Hampton Research, Qiagen, JenaBioscience, Molecular Dimensions). Then optimization was performed by hanging drop vapor diffusion method in Linbro plates at room temperature (293 K). Crystals of L167fs grew over 1 mL reservoir solution (42.5 mM CdSO4, 100 mM Hepes pH 8.0, 960 mM sodium acetate, 3 mM NaNO3) in drops composed of equal volumes (2 μl) of protein solution (8.99 mg/ml in 20 mM Tris-HCl pH 7.4, 0.1 M sodium azide, 0.1M PMSF and 0.1 M EDTA) and reservoir solution. Crystals appeared after 1 or 2 days and grew up to their final size in 1 week. Prior to data collection, single crystals were cryo-protected in the reservoir solution supplemented with increasing concentrations of glycerol up to a final concentration of 35 % (v/v). Data sets were collected at 100 K using synchrotron radiation on beam lines ID23-1 at the ESRF (European Synchrotron Radiation Facility, Grenoble, France). Data were indexed in space group I432, processed with MOSFLM (32), and scaled using SCALA (33) from the CCP4 suite (34). Data collection statistics are given in Table 1.

**Structure determination.** The structure was solved by molecular replacement using the program MOLREP with the atomic coordinates of human L-chain ferritin subunit (35) PDB code: 2ffx. Model building and refinements were performed using the programs COOT (36) and REFMAC5 (37) iteratively. Protein stereo-chemical restraints were taken from (38). Cadmium ions were positioned with the help of anomalous difference Fourier maps. Water molecules were positioned in well-defined positive (mFo-DFc) residual densities with a lower cut-off of 3σ, if they participated in H-bonds with the protein, cadmium ions or other water molecules. The model validity was checked with the program MOLPROBITY (39). The final protein model consists of residues 1-157. Final refinement statistics are given in...
RESULTS

Biochemical characterization of the recombinant L167fs mutant. We expressed the full coding sequence of Lwt and L167fs in E. coli. They were recovered at comparable level in the soluble fraction of the cell extracts (Supplemental Fig. S1). The mutant assembled in 24-mer ferritin shells that were stable at 70°C and could be purified with the same procedure used for Lwt. To study the propensity to form aggregates, L167fs and Lwt were concentrated up to 40 mg/ml and then analyzed by nondenaturing PAGE, they did not show signs of precipitation or evident increase of oligomeric species (Fig. S2B). Also, analyses by Dynamic Light Scattering or Photon Correlation Spectroscopy showed that average hydrodynamic radius (HR) of the mutant (7.39 +/- 0.56 nm) was not significantly different from that of Lwt (7.60 +/- 0.11 nm). To study iron effect on protein aggregation, L167fs and Lwt were incubated with ferrous ammonium sulfate at pH 7.0 for 2 h, separated by nondenaturing PAGE and stained with Coomassie blue or Prussian blue. With increments from 1,000 to 4,000 Fe(II) atoms per ferritin shell we did not observe evident signs of protein precipitation or aggregation, and the amounts of iron incorporated in L167fs and Lwt were equivalent (Fig. 2B). Denaturation studies showed that L167fs was slightly less stable than Lwt (see below). The purified preparations of L167fs contained a smaller peptide of ~19 kDa, the proportion of which increased upon storage at 4°C even in the presence of anti proteases. In a 5-month old preparation it accounted for about 35% of the L167fs (Fig. 2C). This preparation was analyzed by nanoSpray-ESI-MS and showed to contain a 21163 Da species, which corresponds to the full L167fs peptide deleted of the N-terminal methionine (calculated MW= 21164.8 Da), and a minor component of 18921 Da corresponding to the fragment 2-168 (Fig. S2A). The 18 Kd peptide was further analyzed by C-terminal sequencing by MALDI –TOF, obtaining the LGGPEAGLGEYLSS sequence which corresponds to the residues 155-168 of the mutant. This peptide results from cleavage of L167fs after S168, two residues downstream the site of the mutation (Fig. S2B). Lwt is not cleaved under the same conditions, indicating it has a more compact structure, in agreement with the observations that the C-terminus of L167fs is accessible to proteolytic enzymes, while that of Lwt is not (24) and with the evidence that a flagtag epitope attached to the C-terminus of the L154fs mutants is exposed (22). Thus the C-termi of the two mutants have abnormal conformations.

Crystallographic structure of L167fs. For further characterization, the purified protein was crystallized and analyzed by X-ray diffraction and its structure was solved at 1.85 Å resolution. It fully overlapped the Lwt structure from the N-terminus up to residue G157, two residues after the C-terminal end of helix D. Cadmium cations, used in the crystallization conditions were found to bind the protein at the usual metal binding sites, i.e. the residues of the 3-fold axes funnel (H115, C127, D128 and E131) and the nucleation center (H50, E54, E57, E58 and E61). As a whole, the L167fs structure is very close to that of Lwt as well as of the mutant structure determined by Baraibar (25). When superimposing these structures onto that of L167fs, one obtains an rms deviation on Cα positions of 0.36 and 0.29 Å respectively. However, a common feature with the previously published mutant structure (25) is observed: residues G156 and G157 display a similar conformation, which differs from that adopted by the D-E loop in the Lwt structure. This is illustrated in Figure 3A where the four fold axis of the L167fs structure is shown together with the (2mFo-DFc) and (mFo-DFc) electron density maps and the Cα trace of the Lwt structure. The (mFo-DFc) map shows that the residual density at the four-fold axis is very weak and does not allow one to add up any more atoms to the model. Moreover, six well-ordered water molecules have been positioned in direct contact with chains B and D, in place of some side chains of the missing helix E. These observations indicate that there is no disordered chain within the four fold channel. In order to check the integrity of the crystallized protein at the C-terminal part, we performed an SDS-PAGE analysis of the washed and dissolved crystals and of the protein solution used for crystallization (Fig. S4). Both samples migrated to a molecular weight of approximately 21 kDa with a very small quantity of truncated protein detected around 19 kDa. These data altogether suggest that, in the vicinity of the four-fold molecular symmetry axes, the C-terminal part of L167fs is turned outside towards the solvent, as indicated before (25), leading to the disruption of the tightly packed hydrophobic pore, and to the formation of a large permeable opening. This disruption occurs also in heteropolymers in which a single mutant
subunit is present in the four-fold axes as in the models shown in figure 3B. Panels 1 and 2 display the four-fold axis of the L167fs and Hwt structures, respectively. Panel 3 exhibits a model corresponding to three H-chains plus one L167fs chain. The absence of a single helix E in the heteropolymer H/L167fs renders the pore hydrophilic, exposing to the solvent some residues of both L167fs and H-chain E helices. **Hybrid ferritins.** The data showed that L167fs homopolymers do not exhibit major differences from Lwt, except for lower stability due to the disordered structure of the C-terminus. However, this might be not physiologically relevant, since L-ferritin homopolymers are not found inside the cells and the role of the L-chain is to assist the functionality of the H-chain in heteropolymers (1,2). Thus, we produced in vitro hybrid ferritin molecules by renaturing L167fs or Lwt in the presence of H-chain wt. We used a ratio of 1 L-chain with 3-4 H-chains. The efficiency of L167fs assembly was comparable to that of Lwt (not shown), and the electrophoretic mobility of the hybrids was related to the subunit composition (H>H/Lwt>H/L167fs>Lwt>L167fs) (Fig. 4). The renatured ferritins were incubated with 1,000 Fe(II) atoms increments at pH 7.0, separated on nondenaturing PAGE and stained with Prussian blue. Iron incorporation was comparable in all the samples (Fig. 4). However, these conditions are far from the physiological ones, since inside the cell iron is expected to be less available and loosely bound to small molecules such as citrate or phosphate. Therefore we used other conditions that were previously found to distinguish the functionality of H-ferritin mutants with altered permeability at the 3-fold channels (41). The ferritins solutions (0.2 μM) were incubated at pH 7.0 with 0.1 mM 55Fe(III)- citrate complex in the presence of 1 mM ascorbate as reducing agent, and then exposed to autoradiography. Under these conditions the Lwt and L167fs homopolymers did not incorporate radioactive iron, while H-homopolymer was active (Fig. 4). Interestingly, the H/Lwt heteropolymers incorporated radioactive iron, while the H/L167fs heteropolymers did not. It remained to verify that these conditions which distinguish the functionality of H/L167fs heteropolymers are physiologically relevant, and if the same applies to other pathogenic mutants associated with neurodegeneration. **Expression and activity of hybrid ferritins in E. coli.** The study of the functionality of hybrid ferritin molecules in mammalian cells is complex, for example it is still unclear if the L-rich ferritins expressed in the liver are more functional than the H-rich molecules expressed in the heart or brain. Moreover, the analysis of mutant ferritins expressed in transfected cells is confused by the coassembly with the endogenous ferritin subunits. **E. coli** can be useful cellular models, since their endogenous ferritins incorporate iron with a mechanism similar to the one used by mammalian cells and their subunits do not coassemble with the mammalian ones. We made a bicistronic vector (pET-H/Lwt) in which the human H- and L-ferritin cDNAs were consecutively subcloned into a pET vector, downstream of the same, unique promoter. (42). The E. coli were transformed with pET-H, and pET-H/Lwt, and ferritin expression induced. The ferritins were purified and analyzed. The one expressed by the pET-H/Lwt vector contained 90-95% H and 5-10% Lwt which corresponds to about 1-2 L-chains per 24-mer shell (Fig. S5). This ratio is probably close to the one found in the brains, and in particular in the neurons, which contain H-rich ferritins (43). Next we introduced the pathogenic mutations in the L-ferritin, to express L148fs, L154fs, L167fs and also LA96T (Fig. 1). The transformed cells were grown in an iron-poor medium, induced and the cell homogenates heated at 65°C to enrich ferritins. Nondenaturing PAGE showed that the different strains expressed a similar level of the hybrid ferritin shells, and that they had similar electrophoretic mobility. Enhanced Prussian blue staining showed that H-homopolymer and heteropolymers H/Lwt and H/ LA96T incorporated similar amounts of iron, while L-homopolymer bound only trace amounts of iron. More interestingly, the heteropolymers containing the pathogenic mutants L148fs and L154fs incorporated little iron and behaved similarly to Lwt homopolymer. L167fs with the shortest modification of E-helix incorporated slightly more iron but still less than H/Lwt. To study the role of the sequences mutated by the insertions, we produced mutants with a stop codon at the site of the frameshift. The heteropolymers were expressed at the same levels as the other ones and their iron incorporation was as impaired as that of the frameshift mutants, and similar to that of L-homopolymer (Fig. 5A). Staining protein with Coomassie blues or with anti-H and anti-L ferritin monoclonal antibodies confirmed that the loads were properly calibrated and that the samples contained similar proportions of H- and L-chains. As a further verification, the same amounts of the preparations were separated on SDS-PAGE and analyzed by western blotting with a polyclonal antibody with higher affinity for the
L-chain than the H-chain. The dark band of the L-chain had similar intensity in the different heteropolymers, and the deletion mutants had the expected molecular weights (Fig. 5B lower panel). The faster mobility of mutant L148fs may be due to a proline-rich region, which is absent in the other proteins (Fig. 1). Also the paler band of the H-ferritin had similar intensity. Thus, the hybrids contained a similar H:L ratio. In other experiments the different ferritins were separated on nondenaturing PAGE and then initially stained with Coomassie blue to compare the loads (Fig. 5B, upper panel). They were treated for iron removal, and enhanced Prussian blue staining confirmed the absence of iron. The gels containing the proteins were then incubated aerobically either with 200 μM Fe(II) or with 1 mM Fe(III) in the presence of 1 mM ascorbate for 15 min. After the reactions the gels were washed and stained with Prussian blue. The results show that iron incorporation was analogous for the various ferritins when iron was supplied as Fe(II), on the contrary, when Fe(III)-ascorbate was used H-homopolymer, H/Lwt and H/LA96T incorporated a large amount of iron, L-homopolymer was inactive, the heteropolymers with mutations at 154 and 148 (fs or stop) did not incorporate iron and those at 167 had an intermediate activity (Fig. 5B), with an activity sigmoid progression plot (Fig. 6B). Thus the hybrids contained rates of iron release about two fold faster than H/Lwt and H-homopolymers (not shown). This further supports the hypothesis that the mutants make ferritin shells with higher permeability.

**Ferritin stability.** To study protein physical stability, the purified ferritins were treated with 1% SDS at room temperature, and analyzed on discontinuous SDS-PAGE. The Lwt homopolymer released a minor proportion of disassembled subunits (about 5%) while the L167fs more than 20%. Heating at 100°C in 1% SDS fully dissociated both ferritins (Suppl Fig. S6A). Under the same conditions the heteropolymers behaved similarly to the Lwt homopolymers, except the ones containing the mutant with the longest deletion (L148X), which showed a 20% dissociation (Suppl. Fig. S6B). Next we performed unfolding by increasing guanidine hydrochloride concentrations, and monitored the change of intrinsic fluorescence of the single tryptophan (44). The Lwt homopolymer started unfolding at concentrations of 6 M with a midpoint above 8 M, the H-homopolymer had a midpoint at 6.75 M, while the L167fs homopolymer showed a sharp transition between 5 and 6 M, similar to that of H-ferritin (Fig. 7). The H/Lwt heteropolymer plot was intermediate between those of H- and Lwt homopolymers, and those with L154fs and L167fs were shifted to the left. The polymers containing the truncated subunits were the least stable, particularly that with L148X.

**DISCUSSION.**

Hereditary ferritinopathies have similar phenotypic expressions, with brain iron accumulation, movement disorders and the formation of ferritin bodies in the brain and other tissues (15,16,45). However, the ferritin mutations associated with the disorders are rather different: they modify different length of the C-terminus, and one- or two-nucleotide frameshift produce different sequences with extensions of 4 or 16 residues. The characterization of two of these mutants showed remarkably different biochemical properties. While 154fs was hardly soluble, difficult to purify and analyze (23), L167fs was highly soluble, could be easily purified and even crystallized (this work and ref. 24, 25). The
crystals we obtained had a different space group (I432) from that reported in (25) (P1) and diffracted at higher resolution, but the solved structures are very similar. The main common feature of the two structures is the observation of a strong disorganization at the four-fold molecular axis. In the P1 structure, the authors could model 13 chains up to residue Phe165, out of the 144 chains that build the asymmetrical unit. The five last residues G161-F165 point towards the cavity, through the four-fold axis pore. It seems therefore, that such a conformation must be very minority. Moreover, such a chain conformation needs an important rearrangement, in order to construct a heteropolymer model as the one shown on panel 3 of Figure 3B, since the fragment G161-F165 in its actual conformation would overlap with the E helix of a neighboring H-chain (not shown). In our single chain model, the protein chain stops at residue G157, and the absence of residual electron density within the four-fold axis pore, leads us to consider that the C-terminus end is turned outside towards the solvent, certainly a common feature of the two mutants 154fs and 167fs. This characteristic is expected to have two major effects: a reduced physical stability due to the loss of stabilizing interactions around the 4-fold axis, and the formation of a large opening in place of the tight hydrophobic channel, which might affect ferritin functionality. A reduced physical stability of L167fs was demonstrated in the present and previous studies (24). The same probably occurs for the pathogenic mutations with frameshift at residue 148 or 154, where the modified sequences are even longer and interest up to the last two turns of the D-helix, which contain important stabilizing interactions. This could not be explored because of difficulties to obtain sufficient amount of these proteins, due to their folding problems and low solubility. In fact, the H-chain truncated of the last 28 residues (equivalent to 24 residues of the L-chain) could not assemble when expressed in E. coli (21). However, the patients with the L148fs or L154fs showed onset of tremors, gait disturbances and cognitive decline at age earlier than the ones with L167fs (13) suggesting that the upstream insertions have more severe effects than the downstream ones. This may be paradoxical if we consider only the stability/folding properties, since the less stable subunits should have problems to assemble in ferritin shells, and should form homopolymeric aggregates or be degraded. However, the ferritin bodies found in the patients with the L154fs and L167fs mutations were iron-rich and contained H- and L-mutant chains, implying that they are made of assembled shells. Aggregates made only of mutant chains have not been observed in the patients. This apparent contradiction can be explained by the strong tendency of ferritin to form heteropolymers even with subunits with major folding problems. For example L-ferritin mutants with a constructed ferroxidase center were totally insoluble when expressed in E. coli, and could not be renatured in vitro. However, when renatured together with wt H- or L-chains they formed stable heteropolymers that could be analyzed (46). Our data show that all the pathogenic mutants, including the ones with upstream insertions, expressed in E. coli together with H-chain, formed heteropolymers with similar L proportions, and produced soluble and stable ferritins which could be easily purified and analyzed. This occurred also with the truncated mutants, even the ones deleted of the last 28 residues, which were predicted not to assemble when alone. This indicates that in vivo the pathogenic mutants can also assemble with the endogenous ferritin subunits, and that this process is independent from the length of the mutation for these pathogenic mutants. Thus, the pathogenicity of the mutants is more likely due to abnormal properties of heteropolymers containing even a low proportion of the mutant, rather than to the mutant homopolymers. Moreover, the finding that the heteropolymers are highly soluble and present a physical stability only marginally lower than that of the H/Lwt heteropolymers suggests that mutant stability and aggregations are not the primary causes of the disease. It should be noted that the mutants are particularly toxic in the brains and neurons, which are known to have H-rich ferritins with an H/L ratio probably similar to that of the heteropolymers we expressed in E. coli. The in vitro conditions we initially used to study L-homopolymers were not particularly informative: they showed a low solubility of mutant L148fs and a high solubility of L167fs, which did not show signs of aggregation in the absence of iron. In our hands L167fs exhibited only a minor, if any, decrease in solubility when exposed to high iron increments (fig 2B) which contrasts with the data of ref (24,25) showing the massive precipitation of the mutant when incubated with 3-4000 Fe per molecule. Baraibar et al. (25) used a protein with an altered N-terminus, which differs from the native one of our proteins, and this might affect protein solubility. More important, the L-homopolymers lack the ferroxidase activity which is necessary for in vivo iron incorporation (47), thus the in vitro
conditions used to study their functionality are far from the physiological ones. We did not observe the difference in rate of iron oxidation between Lwt and L167fs described in ref (25), and under those conditions the kinetics at 310 nm of the two proteins were much slower and very similar (not shown). The L-chains assist the H-ones in the mechanism of ferritin iron incorporation, thus their functionality must be analyzed when both are together in the same molecule. In fact the heteropolymer study was more informative. It was shown earlier that the reconstituted H/L154fs heteropolymers incorporated in vitro less $^{55}$Fe than H/Lwt, using conditions in which Fe(III) was reduced to Fe(II) by ascorbate (23). The same occurred with the reconstituted H/L167fs heteropolymers, that were incompetent in taking up Fe(III) in the presence of ascorbate, while H/Lwt were active (Fig. 4). A more direct approach to study all the mutants that have been associated with hereditary ferritinopathies was to make the heteropolymers in E. coli and analyze their iron incorporation during expression in the bacteria. This showed that H, H/Lwt and H/LA96T were much more active than the other heteropolymers and Lwt alone. The level of iron incorporation was inversely related to the length of the E-helix modification, with L154fs being less active than L167fs. The same order of iron incorporation was followed by the apoferritins exposed to Fe(III) in the presence of ascorbate. Moreover, the truncated mutants behaved similarly to the frameshift ones, indicating that the presence of an altered sequence at the C-terminus leads to a loss of interactions in the four-fold axis, and thus to the opening of a large pore that modifies the ferritin functionality.

These data demonstrate that mutations on the C-terminal sequence have a dominant negative effect on the ferritin capacity to incorporate iron, and that even 1 or 2 mutant subunits per 24-mer shells are sufficient for this. One subunit is enough to lead to a loss of interactions in the four-fold channels, and thus to the opening of a large pore that modifies the ferritin functionality.

The iron-induced ferritin precipitation occurs at protein ad iron concentrations that are far from the physiological one (25) and has been described for homopolymers but not heteropolymers. Indeed an increase of endogenous ferritin and of iron was found in the brain of the mouse model for ferritinopathy (26). The iron-induced ferritin precipitation is due to the local unfolding of the C-terminus rather than to the properties of the altered sequence, and this consistent with the observation that insertion of one or two nucleotides has similar effects. Our data confirm previous hypothesis that hereditary ferritinopathies are caused by reduced activity of iron incorporation in the ferritin (22). This opens a vicious circle in the cell: the iron not retained by ferritin induces the synthesis of more ferritin chains, both wt and mutant, which form inefficient heteropolymers. Indeed an increase of endogenous ferritin and of iron was found in the brain of the mouse model for ferritinopathy (26). The iron-induced ferritin precipitation occurs at protein ad iron concentrations that are far from the physiological one (25) and has been described for homopolymers but not heteropolymers. Indeed an increase of endogenous ferritin and of iron was found in the brain of the mouse model for ferritinopathy (26).

In conclusion, our data show that the major common property of the pathogenic L-mutants involved in hereditary ferritinopathies is the reduction of iron incorporation activity when assembled in heteropolymers. This reduction is linked to frameshifts or deletions in the C-terminal sequence. The finding that all the mutants act in a dominant negative manner explains the dominant transmission of the disorders. The role of the mutation A96T in neuroferritinopathy should be revised, since it does not have these properties and that does not affect ferritin iron incorporation.

REFERENCES.

FOOTNOTES

ACKNOWLEDGMENTS

This study was partially supported by grants from Telethon-Italia (GGP05141) to S.L. and P.A., and Fondazione CARIPLO-2007 to S.L. and A.C. and by European Community grant Euroiron1 to PA. We thank Dr. Massimo Degano for the Dynamic Light Scattering analyses. We thank the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities and the staff of the ID23-1 beam line for their kind assistance during data collections.
ABBREVIATIONS


FIGURE LEGENDS

Figure 1. Alignment of the C-terminal sequence of the L-ferritin mutants analyzed. Lwt is the sequence of wild type L-ferritin. The amino acid numbering above includes the N-terminal methionine. The C-terminal sequence is conserved in LA96T, since the missense mutation Ala96 Thr is in C-helix. L154fs represents the mutant 460InsA, described in ref (9), L148fs represents the mutant 442InsC described in ref (10). L167fs represent the mutant 498InsTC described in ref (12). L148X, L154X and L167X are the truncated mutants. The mutant produced by 442 4-nucleotide duplication described in ref (13) is the same as L148fs except that the substituted sequence is one amino acid longer. The mutation with a 16 nucleotide duplication in position 469-470 producing a frameshift at residue 162 ref (15) was not analyzed. The D and E alpha helices are indicated by the boxes.

Figure 2. Analysis of the homopolymers of L167fs mutant. A: Nondenaturing PAGE of the purified Lwt and L167fs ferritin homopolymers before (dil) and after concentration to 40 mg/ml (conc). B: Lwt and L167fs were incubated with Fe(II) increments of 1, 2, 3 and 4,000 atoms per molecule (1, 2, 3, 4), at pH 7.0 and then run on nondenaturing PAGE and stained for protein with Coomassie Blue (Co blue) or for iron with Prussian Blue (Pr blue). C: SDS-PAGE of L167fs preparation freshly made (T0) or after 5 months at 4°C. Protein load of 5 µg, representative of three independent experiments with similar results.

Fig 3. Crystal structure of the L167fs mutant. A: Stereo view of two opposite chains of the four-fold axis of L167fs mutant structure (shown as balls and sticks) together with the (2mFo-DFc) electron density map (blue) contoured at 1.0 σ and the (mFo-DFc) electron density map (pink) contoured at 3.0 σ. The Cα trace of the Lwt structure is superimposed and shown in black. B: Four-fold axes representation: (1) L167fs crystal structure (yellow), (2) human H-chain crystal structure and (3) a heteropolymer model composed of one L167fs chain (yellow) and three H-chains. The surfaces represent the solvent accessible areas. In panel (2), the residues represented as sticks are those which line the four-fold axis. In panel (3), the residues represented as sticks are those which polar atoms have their solvent accessible surface increased due to the absence of the C-terminal part of the L167fs subunit within the pore, i.e. A44, L45, G47, T96, H148 and L152 for the L167fs subunit, and N154, K157, M158, H173, Y168, K172, H173 for the H-chain subunits that are in close contact with the L167fs subunit.

Fig. 4. In vitro assembled ferritin heteropolymers. The purified ferritins were denatured and renatured alone to produce the Lwt, Hwt or L167fs homopolymers, or renatured together to produce the H/Lwt and H/L167fs heteropolymers with H:L ratio of 3:1. The protein were incubated with 1,000 Fe(II) atoms at pH 7.0, 5 µg run on nondenaturing PAGE and stained for protein (Co Blue) or with Prussian blue (Pr Blue). Alternatively they were incubated with radioactive 55Fe(III) in the presence of 1 mM ascorbate at pH 7.0, 1.5 µg loaded on nondenaturing PAGE and exposed to autoradiography for iron incorporation detection (Autoradiography). Representative of three independent experiments with similar results.

Figure 5. Analysis of the heteropolymers with the pathogenic and truncated L-subunits expressed by E.coli. A: The E. coli transformed with the bicistronic vector to express the heteropolymers were grown in iron poor medium and the homogenates heated at 65°C for ferritin enrichment. The protein were run on nondenaturating PAGE and stained with enhanced Prussian blue (E. Pr Blue), Coomassie blue (Co Blue) or Western blotted (WB) and stained with monoclonal antibodies for L-ferritin (Anti-FTL) or H-ferritin (Anti-FTH). The bottom panel shows an SDS-PAGE blotted with a polyclonal antibody that has higher affinity for the L-chain (darker bands), but that recognizes also H-chains (paler bands indicated with an asterisk). The + above the lanes indicates the molecules containing H-chain. B: The ferritins were separated on nondenaturating PAGE and stained with Coomassie blue (Co Blue). Then they were treated for iron removal and colored with enhanced Prussian blue to verify the absence of iron (E. Pr Blue). The gels containing the apoproteins were incubated for 15 min with 200 µM Ferrous ammonium sulfate at pH 6.5 (Fe(II) then washed and stained with Prussian blue (Pr Blue) or were incubated for 15 min with 1 mM ascorbate and 1
mM Ferric ammonium citrate in 20 mM Tris HCl pH 7.0 (Fe(III)-Asc), extensively washed and stained with enhanced Prussian blue (E. Pr Blue). Representative of three independent experiments.

**Figure 6. Ferroxidase activity of the ferritin heteropolymers.** A: the purified apoferritins (0.1 μM) were incubated with 0.1 mM ferrous ammonium sulfate at pH 6.5 and the iron oxidation reaction followed at 310 nm. B: the apoferritins (0.2 μM) were incubated with 4 mg/ml apotransferrin, in 0.2 M sodium acetate pH 6.0, supplemented with 1 mM ferrous ammonium sulfate, and the formation of pink holotransferrin followed at 460 nm. Representative of three independent experiments.

**Figure 7. Unfolding plots in guanidine chloride of the purified heteropolymers.** The ferritins (50 μg/ml) were incubated for 18 h at 4°C in 0.1 M phosphate buffer, pH 7.4, 1 mM DTT, with the different guanidine-HCl concentrations. Unfolding was determined by fluorescence spectroscopy (excitation at 295 nm), using, as standards, ferritin either in 0.1 M phosphate, pH 7.4, (native state) or in 0.1 M phosphate, pH 3.0 and 6 M guanidine-HCl (denatured state). Data were plotted as fraction of unfolded shells. H, Lwt and L167fs are the corresponding homopolymers, H/Lwt, H/L154fs, H/L167Fs, H/L148X and H/L154X are the heteropolymers. Representative of three independent experiments.
Table 1: X-ray diffraction data collection and refinement statistics

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>I432</td>
</tr>
<tr>
<td><strong>Cell parameter a (Å)</strong></td>
<td>151.355</td>
</tr>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>61.78 - 1.85 (1.95 - 1.85)</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>25503 (3572)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.3 (98.3)</td>
</tr>
<tr>
<td>( R_{\text{merge}} ) (%)</td>
<td>0.075 (0.407)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.1 (5.2)</td>
</tr>
<tr>
<td>(&lt;\text{I}/\sigma&gt;)</td>
<td>5.8 (2.0)</td>
</tr>
<tr>
<td><strong>Model and refinement</strong></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>61.78 - 1.85 (1.90 - 1.85)</td>
</tr>
<tr>
<td>Number of used reflections</td>
<td>23733 (1706)</td>
</tr>
<tr>
<td>Number of non-hydrogen atoms</td>
<td>1502</td>
</tr>
<tr>
<td>Mean B values (Å²)</td>
<td>17.97</td>
</tr>
<tr>
<td>RMSD bond lengths (Å)</td>
<td>0.014</td>
</tr>
<tr>
<td>RMSD bond angles (°)</td>
<td>1.24</td>
</tr>
<tr>
<td>( R_{\text{work}} ) (%)</td>
<td>0.179 (0.226)</td>
</tr>
<tr>
<td>( R_{\text{free}} ) (%)</td>
<td>0.211 (0.316)</td>
</tr>
<tr>
<td>Ramachandran plot statistics (%)</td>
<td></td>
</tr>
<tr>
<td>Favored regions</td>
<td>98</td>
</tr>
<tr>
<td>Allowed regions</td>
<td>100</td>
</tr>
</tbody>
</table>

Values in parentheses correspond to the highest resolution shell.

\[ R_{\text{merge}} = \frac{\sum_{h} \sum_{i} |I_{h,i} - \langle I_{h} \rangle|}{\sum_{h} \sum_{i} I_{h,i}} \text{ where } \langle I_{h} \rangle \text{ is the average of symmetry related observations for unique reflections.} \]

\[ R_{\text{work}} = \frac{\sum_{h} |F_{h}^{\text{obs}} - F_{h}^{\text{calc}}|}{\sum_{h} F_{h}^{\text{obs}}} \text{, where } F_{h}^{\text{obs}} \text{ and } F_{h}^{\text{calc}} \text{ are the observed and calculated structure factor amplitudes, respectively.} \]

\[ R_{\text{free}} = \text{ as for } R_{\text{work}}, \text{ but for 5\% of the total reflections chosen at random and omitted from refinement.} \]
Figure 1

141  175

L-wt  LIKEMSDELNLRLGPEARGLEYLFERTLKLHD
L-A96T LIKEMSDELNLRLGPEARGLEYLFERTLKLHD
L-148fs  ********PPDPQPGWPGWNAGRVSFAHSQARLRAF
L-154fs  **********KAQWPGWNGRVSFAHSQARLRAF
L-167fs  ******************SSKGSLSSTRTLSPATSEGPLAK
L-148X  LIKEMS
L-154X  LIKEMSDELNLHR
L-167X  LIKEMSDELNLRLGPEARGLEYL

D-Helix  E-Helix
Figure 5A
| H chain | + | + | + | + | + | + | + | + | + | - |
| L chain | - | wt | A96T | 154fs | 148fs | 167fs | 167x | 154x | 148x | wt |

---

**Figure 5B**

- Co blue
- E. Pr blue
- Pr blue
- E. Pr blue
Figure 6A
Figure 6B
Figure 7
MUTANT FERRITIN L-CHAINS THAT CAUSE NEURODEGENERATION ACT IN A DOMINANT NEGATIVE MANNER TO REDUCE FERRITIN IRON INCORPORATION

Sara Luscieti, Paolo Santambrogio, Beatrice Langlois d'Estaintot, Thierry Granier, Anna Cozzi, Maura Poli, Bernard Gallois, Dario Finaazzi, Angela Cattaneo, Sonia Levi and Paolo Arosio

J. Biol. Chem. published online February 16, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M109.096404

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/02/16/M109.096404.DC1