

# Hereditary hemochromatosis: Effects of C282Y and H63D mutations on association with $\beta_2$ -microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells

(degradation/Golgi processing/intracellular transport/iron/major histocompatibility complex class I protein)

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**ABSTRACT** Hereditary hemochromatosis (HH) is the most common autosomal recessive disorder known in humans. A candidate gene for HH called HFE has recently been cloned that encodes a novel member of the major histocompatibility complex class I family. Most HH patients are homozygous for a Cys-282→Tyr (C282Y) mutation in HFE gene, which has been shown to disrupt interaction with  $\beta_2$ -microglobulin; a second mutation, His-63→Asp (H63D), is enriched in HH patients who are heterozygous for C282Y mutation. The aims of this study were to determine the effects of the C282Y and H63D mutations on the cellular trafficking and degradation of the HFE protein in transfected COS-7 cells. The results indicate that, while the wild-type and H63D HFE proteins associate with  $\beta_2$ -microglobulin and are expressed on the cell surface of COS-7 cells, these capabilities are lost by the C282Y HFE protein. We present biochemical and immunofluorescence data that indicate that the C282Y mutant protein: (i) is retained in the endoplasmic reticulum and middle Golgi compartment, (ii) fails to undergo late Golgi processing, and (iii) is subject to accelerated degradation. The block in intracellular transport, accelerated turnover, and failure of the C282Y protein to be presented normally on the cell surface provide a possible basis for impaired function of this mutant protein in HH.

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder characterized by iron overload of parenchymal cells in many organs including the liver, pancreas, heart, joints, and endocrine organs due to increased iron absorption in the gastrointestinal tract (1–4). Clinical consequences of iron accumulation in these organs include cirrhosis of the liver, hepatocellular carcinoma, diabetes, heart failure, arthritis, and hypogonadism. Within the Caucasian population, 1 in 300–400 individuals is homozygous and 1 in 8–10 individuals is heterozygous for HH (3, 5). Recently, Feder *et al.* (6) reported that 83% of 178 American HH patients were homozygous for the same missense mutation (C282Y) in a novel major histocompatibility complex (MHC) class I-like gene, originally called HLA-H, but now designated HFE (7). [Although Feder *et al.* (6) originally designated the HH candidate gene HLA-H, this designation had already been assigned to a pseudogene and the HH locus had already been assigned the name HFE by the nomenclature committee (7).] Eight of nine patients with HH who were heterozygous for this mutation were found to

have a different missense mutation (H63D) on the other HFE allele, although 17% of the normal population also carried one H63D allele. These findings were confirmed by Beutler *et al.* (8), who found that 82% of 147 HH patients were homozygous for the C282Y mutation and 10 were compound heterozygotes for C282Y and H63D alleles. Subsequent studies reported that 72–91% of French patients (9, 10), 64% of Italian patients (11), and 100% of Australian patients (12) were homozygous for the C282Y mutation. Independent support for HFE as the HH gene comes from the observation that the  $\beta_2$ -microglobulin ( $\beta_2$ M) knockout mouse shows evidence of iron overload similar to that seen in HH patients, which suggested the possible involvement of an MHC class I gene in HH (13, 14).

The exact mechanisms of iron absorption and how the HFE protein could regulate it are unknown. However, Parkkila *et al.* (15) showed recently by immunohistochemistry that the HFE protein is expressed in certain epithelial cells throughout the human alimentary tract and has a unique localization in the small intestine, where signals to alter iron absorption are received from the body and these signals are translated into an absorption response after the cells have differentiated into mature absorptive enterocytes (16). These observations suggested a possible role for HFE protein in regulating iron absorption in the small intestine.

The human HFE protein predicted from the cDNA sequence is composed of 343 amino acids. Database comparisons revealed that it is most homologous to MHC class I molecules, which contain an extracellular peptide binding region ( $\alpha 1$  and  $\alpha 2$  loops), an Ig-like domain ( $\alpha 3$ ), a transmembrane region, and a short cytoplasmic tail. By analogy with other MHC class I proteins, HFE is presumed to contain intramolecular disulfide bridges in the  $\alpha 2$  and  $\alpha 3$  domains that stabilize its tertiary structure (6). It had been suggested that the disulfide bridge in the  $\alpha 3$  domain of MHC class I molecules is required for their association with  $\beta_2$ M, leading to efficient intracellular processing and transport to the plasma membrane (17, 18). In fact, Feder *et al.* (19) recently demonstrated failure of the C282Y HFE protein to associate with endogenous  $\beta_2$ M in 293 cells stably transfected with the mutant cDNA. The aims of this study were to determine the effects of the C282Y and H63D mutations on the cellular trafficking and degradation of the HFE protein in transfected COS-7 cells. The results indicate that, while the wild-type and H63D HFE proteins associate with coexpressed  $\beta_2$ M and are expressed on the cell surface, these capabilities are lost by the C282Y HFE protein. Much of

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Abbreviations:  $\beta_2$ M,  $\beta_2$ -microglobulin; endo-H, endoglycosidase H; ER, endoplasmic reticulum; HH, hereditary hemochromatosis; MHC, major histocompatibility complex.

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the C282Y protein remains in high molecular weight aggregates, and fails to undergo late Golgi processing or be expressed on the cell surface. Over half of the newly synthesized C282Y protein undergoes accelerated degradation before processing in the middle Golgi. These abnormalities in intracellular processing and cell surface presentation of the mutant HFE protein may provide a mechanism to explain how the C282Y mutation might lead to loss of function of the HFE protein.

## MATERIALS AND METHODS

**Antibodies.** Based on the structure of the HFE protein derived from the cDNA sequence, three different antisera for peptides of HFE protein were produced in rabbits. The production and characterization of the antibody raised against a polypeptide of 16 C-terminal amino acids has been described (15). We refer to this antibody as CT16. Antibodies to two peptides predicted for the extracellular domains (amino acids 164–177 of the  $\alpha 2$  domain and amino acids 246–260 of the  $\alpha 3$  domain) were the same antibodies recently described as EX1 and EX2, respectively (19). Using corresponding peptide-Affigel 10 affinity resins, affinity-pure and peptide-specific IgGs were isolated and stored in 50% glycerol at  $-20^{\circ}\text{C}$ . Rabbit anti-human  $\beta_2\text{M}$  antibody was purchased from Sigma.

**Transfection of COS-7 Cells.** The missense mutants at residue 282, C282Y and at residue 63, H63D of the HFE cDNAs were constructed by site-directed mutagenesis using mismatched primers. The procedure for generating mutants was based on the protocol of Deng and Nickoloff (20). Both wild-type and mutant cDNAs of HFE and cDNA of human  $\beta_2\text{M}$  were subcloned into the pCAGGS vector as described (21). COS-7 cells plated on chamber slides for microscopy or on p-35 or p-60 plates were transfected with 1, 3, and 10  $\mu\text{g}$  cDNA, respectively, using the DEAE-dextran procedure (22) followed by 1.5 h incubation with 100  $\mu\text{M}$  chloroquine (23). When COS-7 cells were cotransfected with human  $\beta_2\text{M}$  cDNA, 3–5  $\mu\text{g}$  of HFE cDNA and 3–5  $\mu\text{g}$  of  $\beta_2\text{M}$  cDNA were mixed. For the control experiments COS-7 cells were transfected with pCAGGS vector alone.

**Homogenization and Subcellular Fractionation of COS-7 Cells.** The COS-7 cells and medium were collected 72 h after the transfections and the cells were homogenized by ultrasonication in ice-cold 50 mM sodium phosphate buffer (pH 7.5), containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM *o*-phenanthroline as protease inhibitors. The cell homogenates were centrifuged at  $135,000 \times g$  for 30 min. The cytosol and membrane pellets were recovered and the membrane pellets were suspended in the homogenization buffer.

**Chemical Crosslinking and Affinity Purification of HFE Protein- $\beta_2\text{M}$  Complex.** The membrane suspension of COS-7 cells transfected with HFE and  $\beta_2\text{M}$  cDNAs was mixed with reversible bifunctional crosslinker, 1 mM dithiobis (succinimidyl propionate) in 50 mM sodium phosphate buffer (pH 7.5), containing the protease inhibitors. The mixture was incubated at room temperature for 10 min. The crosslinking was quenched with 10 mM ethanolamine. The crosslinked proteins were solubilized with 1% Nonidet P-40 and the membrane extract was recovered after centrifugation at  $40,000 \times g$  for 1 h. The membrane extracts obtained from crosslinked membranes were applied to an anti-HFE-CT16 IgG Affigel-10 column. Unbound proteins were removed by washing with PBS. The bound proteins were eluted with 0.1 M glycine-HCl solution (pH 2.5) containing 0.1% Nonidet P-40. The eluted material was mixed appropriately with 1 M Tris base to achieve neutral pH. The eluted proteins were concentrated using Centricon P-10 tubes (Amicon, Beverly, MA) and analyzed by SDS/PAGE under reducing conditions followed by Western blot analysis.

**SDS/PAGE and Western Blot Analysis.** SDS/PAGE was performed under reducing conditions in a Mini-Protean electrophoresis unit (Bio-Rad) according to Laemmli (24). The polypeptides were transferred electrophoretically from the gel to a polyvinylidene difluoride membrane (Millipore), and immunostaining was performed using anti-HFE and  $\beta_2\text{M}$  antibodies followed by incubation with peroxidase-conjugated goat anti-rabbit antibody (Sigma). The peroxidase activity was visualized using a chemiluminescent substrate.

**Metabolic Labeling and Immunoprecipitation.** COS-7 cells were metabolically labeled 48 h after transfections with  $^{35}\text{S}$ -translabel, 50  $\mu\text{Ci}$  (1 Ci = 37 GBq) per p-35 plates, in 1 ml of DMEM (without cysteine and methionine) containing dialyzed and heat-inactivated fetal calf serum as described (21). In pulse-chase experiments, the cells were metabolically labeled for 30–60 min and chased with cold 10 mM cysteine and 10 mM methionine for the required time.

The cells were harvested and lysed in 1 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.1% benzamidine. After sonication of the cell homogenates, 50  $\mu\text{l}$  of 20% IgGSORB (The Enzyme Center, Malden, MA) was added, followed by mixing for 1 h at  $4^{\circ}\text{C}$ . The cell extract was recovered by centrifugation at  $39,000 \times g$  for 30 min. In the biosynthesis experiments, total trichloroacetic acid-precipitable counts were determined and an equal number of counts used for immunoprecipitation at each time point. Affinity-purified CT16 antibody (10  $\mu\text{g}$ ) was mixed with the cell extract for 16 h at  $4^{\circ}\text{C}$ . The immunocomplexes were recovered by adding 80  $\mu\text{l}$  20% IgGSORB, mixing for 1 h at  $4^{\circ}\text{C}$ , and centrifuging at  $15,000 \times g$  for 1 min. The immunocomplexes were washed as described (21) and solubilized in Laemmli's sample buffer (24) for SDS/PAGE analysis.

**Endoglycosidase H (Endo-H) Treatment.** Each immunocomplex was dissociated with endo-H solubilization buffer containing 33 mM Na-phosphate (pH 5.4), 0.66% SDS, and 3.3 mM DTT by heating the sample at  $98^{\circ}\text{C}$  for 6 min. The solubilized sample was recovered after centrifugation and diluted 10 times with 28 mM Na-phosphate buffer (pH 5.4), containing 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine as protease inhibitors. The sample was divided in two equal parts, one of which was treated with 4 milli-enzyme units (mEU) endo-H and the other treated with buffer alone (at  $37^{\circ}\text{C}$  for 24 h). The endo-H reaction was quenched with an equal volume of  $2\times$  Laemmli's sample buffer (24) and analyzed by SDS/PAGE, followed by fluorography.

**Immunocytochemical Staining.** The COS-7 cells transfected with HFE and  $\beta_2\text{M}$  cDNAs were fixed with 4% paraformaldehyde in PBS for 15 min. After rinsing with PBS, they were subjected to immunofluorescence staining on chamber slides that consisted of the following steps: (i) blocking with 1:10 diluted cow colostrum in PBS for 40 min and rinsing in PBS, (ii) incubation for 1 h with a mixture of affinity-purified primary antibodies (10  $\mu\text{g}$  of CT16 IgG and 25  $\mu\text{g}$  of EX1 and EX2 IgG in 500  $\mu\text{l}$  of 0.1% BSA in PBS), and (iii) incubation for 1 h with 1:100 diluted rhodamine-conjugated goat anti-rabbit IgG antibodies (Dakopatts, Glostrup, Denmark) in 0.1% BSA-PBS. The specimens were washed three times for 10 min after the incubation steps. In some cases, the cells were permeabilized using 0.05% saponin. The cells were viewed with a conventional epifluorescence microscope (Zeiss Axio-plan, Zeiss) and a confocal laser scanning microscope (Zeiss Axiovert 135 microscope combined with a LSM 410 CLSM system). The specimens were excited with a laser beam at a wavelength of 568 nm and the emission light was focused through a pinhole aperture. The full field was scanned in square image formats of  $512 \times 512$  pixels and built-in software was used to reconstruct the images obtained from the confocal sections.

## RESULTS

**Expression and Association of HFE Protein with  $\beta_2$ M in COS-7 Cells.** To characterize further the effects of the HH mutations on the properties of the HFE protein, we studied the expression of wild-type HFE protein in transfected COS-7 cells with and without cotransfected human  $\beta_2$ M cDNA. Fig. 1A shows Western blots of extracts from transfected COS-7 cells probed with antibodies to the C-terminal peptide of HFE protein (CT16) and to human  $\beta_2$ M. The HFE protein was identified by the CT16 antibody as a doublet of 48- and 45-kDa polypeptides in COS-7 cells transfected with HFE cDNA (lane

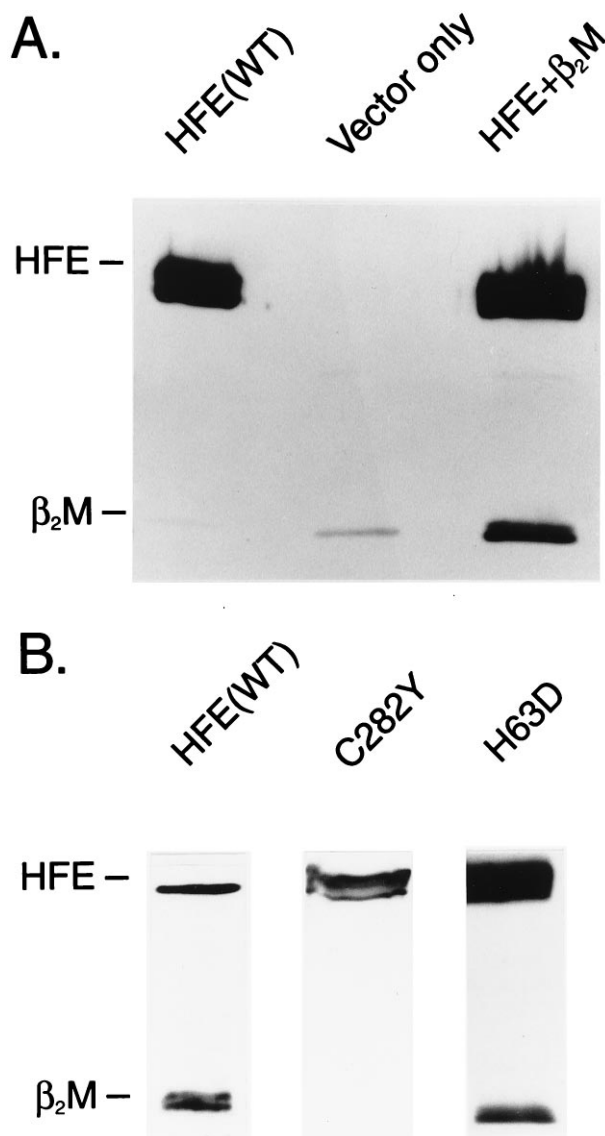


FIG. 1. Expression of HFE protein and association with  $\beta_2$ M. (A) Western blot of HFE and  $\beta_2$ M in transfected COS-7 cells. Homogenates from cells transfected with HFE cDNA alone [HFE(WT)], vector only, or HFE +  $\beta_2$ M cDNAs were analyzed by SDS/PAGE followed by immunostaining using both CT16 and  $\beta_2$ M antibodies. The bands corresponding to HFE protein and  $\beta_2$ M are marked. (B) Western blots of HFE proteins and associated  $\beta_2$ M. Complexes crosslinked by the reversible crosslinker in membranes from cells cotransfected with  $\beta_2$ M cDNA and cDNAs for the wild-type HFE, C282Y HFE, H63D HFE were retained on a CT16 IgG Affigel-10 column and eluted with glycine-HCl, pH 2.5, buffer, and analyzed by SDS/PAGE under reducing conditions. Note that the affinity-purified C282Y mutant protein had no associated  $\beta_2$ M. By contrast, the wild-type and H63D mutant proteins both were isolated in complexes containing crosslinked  $\beta_2$ M.

1), but no immunoreactive HFE protein was detected in cells transfected with vector only (lane 2). However, the endogenous  $\beta_2$ M polypeptide was detected in COS-7 cells (lanes 1 and 2). Lane 3 of Fig. 1A shows that the signal for  $\beta_2$ M was increased 10- to 20-fold over that of the endogenous  $\beta_2$ M, when the COS-7 cells were cotransfected with cDNAs for HFE and human  $\beta_2$ M.

Fig. 1B presents a Western blot of wild-type and mutant HFE proteins and  $\beta_2$ M separated on reducing SDS/PAGE gels following isolation of crosslinked membrane protein complexes by the anti-HFE-CT16 IgG Affigel-10 column. The Western blot shows that both the wild-type and H63D HFE proteins expressed in COS-7 cells are associated with the coexpressed  $\beta_2$ M, as indicated by the fact that they copurify with  $\beta_2$ M in a cross-linked complex on the anti-HFE affinity column. By contrast, no  $\beta_2$ M is cross-linked with the C282Y HFE protein isolated under identical conditions. These results indicate that the C282Y mutation prevents the association between  $\beta_2$ M and HFE protein when coexpressed in COS-7 cells.

**Effects of the C282Y Mutation on the Intracellular Transport and Cell Surface Expression of HFE Protein.** To study the rate of intracellular transport, we carried out metabolic labeling for 30 min and analyzed the immunoprecipitated HFE protein for endo-H resistance after various chase periods. The results in Fig. 2 provide evidence that some of the C282Y mutant protein does move from the ER to the middle Golgi compartment (as evidenced by acquisition of endo-H resistance), but it is transported more slowly and less completely than the wild-type and H63D mutant proteins (Fig. 2 Lower).

The effect of the C282Y mutation on the cell surface expression of HFE protein in transfected COS-7 cells was studied using immunocytochemistry. Fig. 3 shows the results of immunostaining of COS-7 cells cotransfected with  $\beta_2$ M

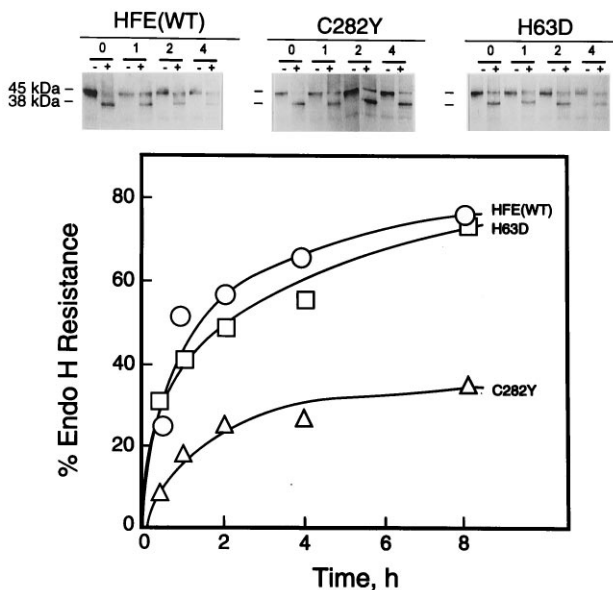


FIG. 2. Effect of C282Y and H63D mutations on the rate of intracellular transport of HFE proteins from ER to middle Golgi. After 60 h from the start of transfection COS-7 cells were pulse-labeled for 30 min with  $^{35}$ S-translabel and then chased for 1–8 h with nonradioactive methionine and cysteine. Immunoprecipitates containing HFE proteins at each time point were incubated with (+) or without (-) endo-H. Products of the deglycosylation reactions were analyzed by SDS/PAGE followed by fluorography. The results were quantitated by calculating the percentage of endo-H-resistant HFE protein at each time point. Note the delay in acquisition of endo-H resistance of the C282Y protein compared with wild-type and H63D proteins. (The lanes from the 8 h time points are not shown in the fluorogram.)

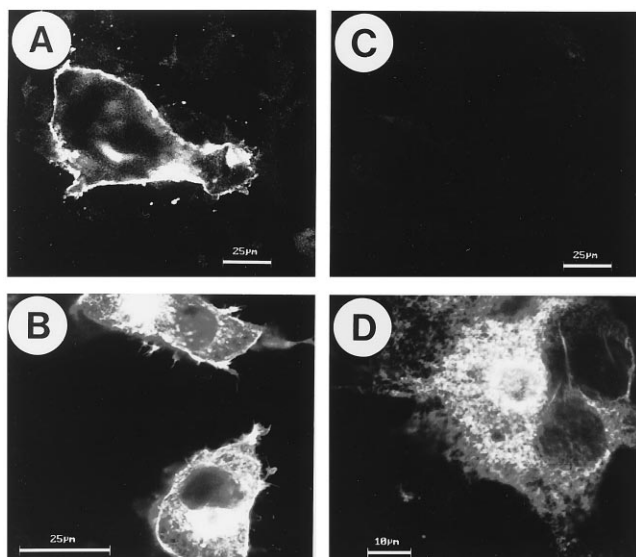


FIG. 3. Immunocytochemistry of HFE protein in COS-7 cells cotransfected with wild-type HFE (A and B) or C282Y mutant (C and D) and  $\beta_2M$  cDNAs. In nonpermeabilized cells, a strong positive immunoreaction is seen in the plasma membrane of the cells transfected with wild-type HFE cDNA (A), but the signal for HFE on the cell surface is below the detection limit in cells transfected with the C282Y cDNA (C). In the permeabilized COS-7 cells, the wild-type HFE protein is seen in both ER and Golgi (B), while the signal for the C282Y mutant protein is predominantly in ER (D).

cDNA, and either wild-type (Fig. 3A and B) or C282Y mutant (Fig. 3C and D) HFE cDNAs. Comparison of the cell surface staining of nonpermeabilized cells in Fig. 3A and C shows that the wild-type HFE protein is highly expressed on the cell surface, but cell surface signal for C282Y mutant protein is below the limit of detection. Fig. 3B and D show the immunostaining of cells from the same transfections that were permeabilized to allow staining of the intracellular wild-type and C282Y HFE proteins. Note the perinuclear, "Golgi-like" and the reticular, "endoplasmic reticulum (ER)-like" distributions of the wild-type HFE protein in Fig. 3B, and the predominantly "ER-like" distribution of the C282Y protein in Fig. 3D, suggesting substantially greater ER retention of the C282Y mutant protein. The cell surface staining of the expressed H63D mutant HFE protein was similar to that of the wild-type HFE protein (data not shown).

**Biosynthesis and Turnover of the HFE Protein.** To compare the rates of biosynthesis of wild-type and mutant HFE proteins, we measured the incorporation of  $^{35}S$ -translabel into the immunoprecipitable protein over a 4-h period. Incorporation was linear over the first 2 h, at which time 2% of the total trichloroacetic acid-precipitable counts was in HFE protein, and tapered off over the next 2 h (Fig. 4). The rates of synthesis of C282Y and H63D mutant proteins were similar to the rate of synthesis of wild-type HFE protein. The apparent retention of some of the C282Y mutant protein in the ER raised the possibility of accelerated turnover of the C282Y protein, as many mutant proteins that have impaired egress from the ER undergo accelerated turnover (25). Fig. 5 presents results of a pulse-chase experiment in which the transfected COS-7 cells were labeled during a 30-minute pulse and chased for 1–8 h. The radioactive polypeptides from the immune precipitates from COS-7 cells transfected with the wild-type, C282Y, and H63D HFE cDNAs all ran at 45 kDa at early timepoints. The polypeptide band became more diffuse between 45–48 kDa during the 1–8 h chase periods in the case of the wild-type and H63D transfections. However, the C282Y mutant protein persisted as predominantly a 45-kDa polypeptide band over the 8-h chase. Fig. 5 Lower shows the quantitation of the

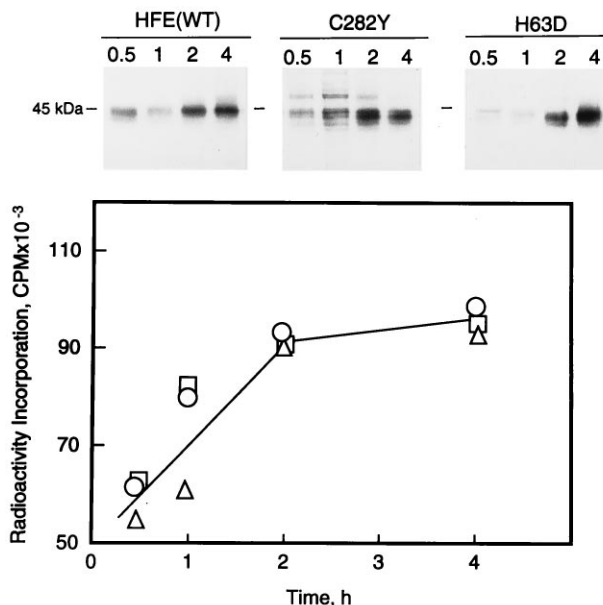


FIG. 4. Biosynthesis of HFE proteins in COS-7 cells. After 60 h from the start of transfection, cells were pulse-labeled with  $^{35}S$ -translabel for 0.5–4 h. At each time point, equal amounts of trichloroacetic acid-precipitable counts from cell homogenate were subjected to immunoprecipitation using CT16 IgG. The immunoprecipitates for wild type (○), C282Y (△), and H63D (□) were analyzed by SDS/PAGE followed by fluorography. The fluorogram was quantitated by cutting and counting the gel pieces corresponding to the HFE bands. The quantitative results are shown (Lower).

pulse-chase experiments. In the first 4 h, the radioactivity in immunoprecipitable wild-type protein and H63D mutant protein decreased by  $\approx 20\%$ , whereas that in the C282Y mutant

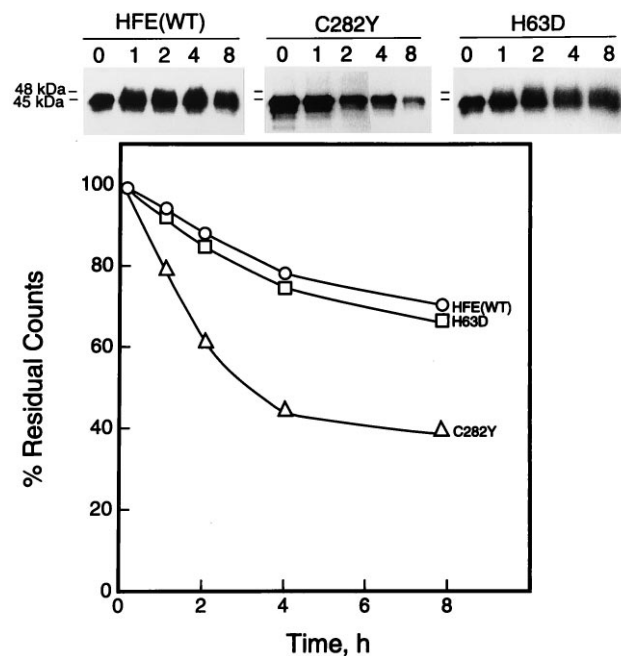


FIG. 5. Effect of C282Y mutation on the rate of degradation of HFE protein in COS-7 cells. After 60 h from the start of transfection, the cells were pulse-labeled for 30 min (○) with  $^{35}S$ -translabel and chased with nonradioactive methionine and cysteine for 1–8 h. The HFE protein was immunoprecipitated and analyzed by SDS/PAGE followed by fluorography. The quantitative analysis of the fluorogram is presented in the lower panel. Note that turnover of C282Y mutant is initially faster than that of the wild-type and H63D mutant proteins.

protein decreased >50%. In the next 4 h, the turnover rate for all three proteins was slower and the loss of label was about 2%/h for all three proteins. These results demonstrate that the C282Y mutation has no effect on the rate of biosynthesis of the HFE protein, but it does affect the rate of posttranslational processing and also increases the rate of degradation of a large fraction of the newly synthesized mutant protein.

## DISCUSSION

C282 is one of four conserved cysteine residues in MHC-class I molecules and forms an important disulfide bond in the  $\alpha 3$  domain (17, 26, 27). The structural element to which this disulfide bond contributes has been suggested to play a role in  $\beta_2$ M association with the  $\alpha 3$  domain (27), and this association with  $\beta_2$ M considered to be necessary for intracellular processing, transport, and cell surface expression of MHC class I molecules (18, 28, 29). In an earlier study on mouse H-2L<sup>d</sup> where site-directed mutagenesis of C203S abolishes the analogous disulfide bond between C203 and C259 of the  $\alpha 3$  domain, the mutant protein was not expressed on the cell surface. However, the mutant H-2L<sup>d</sup> was reported to be glycosylated and able to associate with  $\beta_2$ M (18). The results presented here and those of Feder *et al.* (19) clearly show that the C282Y mutation abrogates the association of the mutant HFE protein with  $\beta_2$ M. This could mean that the mutation of C203S in H-2L<sup>d</sup> (which is equivalent to C225 of HFE) is less deleterious to the structural requirement for association with  $\beta_2$ M than the mutation of C282Y in HFE (which is equivalent to C259 in H-2L<sup>d</sup>). Alternatively, the disulfide bridges in the  $\alpha 3$  domain for  $\beta_2$ M association may be less stringent for H-2L<sup>d</sup> than for the HFE molecule. Nonetheless, one important experimental result of the present study agrees well with the earlier studies of mouse H-2L<sup>d</sup> protein (18) and mutant HFE protein (19), namely that disruption of the disulfide bond in the  $\alpha 3$  domain resulted in a block in intracellular transport and cell surface expression of the C282Y mutant protein.

The subcellular location at which the transport of mutant HFE (19) or H-2L<sup>d</sup> (18) is blocked was not clear from the prior studies. The present study clarifies the sites at which the intracellular transport is impaired. The newly synthesized HFE polypeptide is a cotranslationally *N*-glycosylated protein with a molecular mass of 45 kDa. Complete deglycosylation of the polypeptide with endo-H reduces it to 38 kDa, the size predicted from the cDNA sequence for the nonglycosylated polypeptide after removal of the N-terminal leader sequence (6). The intermediates following partial endo-H digestion (data not shown) suggest that the endo-H removes three N-linked oligosaccharide chains. The carbohydrate of HFE is further processed in COS-7 cells in the middle Golgi compartment (30), resulting in a 48-kDa endo-H-resistant polypeptide. Overexpression of human  $\beta_2$ M in COS-7 cells expressing wild-type HFE protein allows much more of the expressed HFE to be transported to the late Golgi compartments where galactosylation and sialylation normally occur (31). Terminal processing of the oligosaccharides results in a 49-kDa polypeptide. The late Golgi processing and the transport of the bulk of the overexpressed wild-type HFE to the cell surface were both clearly dependent on overexpression of  $\beta_2$ M in COS-7 cells (Fig. 1A and Fig. 5).

The results of studies of the biosynthesis of C282Y mutant protein show that the initial product is also a 45-kDa glycosylated polypeptide. As with the wild-type HFE protein, complete deglycosylation of the 45-kDa polypeptide from the C282Y mutant protein reduces it to a 38-kDa protein. Pulse labeling data showed that the transit of the endo-H-sensitive C282Y mutant protein from ER to middle Golgi was slower than that seen for the wild-type HFE, as evidenced by the slower rate of acquisition of endo-H-resistant oligosaccharides in the middle Golgi (30). The products identified on Western

blots at steady state (72 h after transfection) suggest that the oligosaccharides of nearly 50% of the C282Y mutant protein are eventually processed, probably in the middle Golgi compartment, resulting in a 48-kDa polypeptide (data not shown). However, unlike wild-type HFE, very little of the C282Y mutant protein expressed in COS-7 cells undergoes further processing to the 49-kDa form, regardless of the extent of overexpression of the  $\beta_2$ M.

Much of the newly synthesized C282Y mutant HFE protein remains in a high molecular weight aggregate that can be dissociated on SDS/PAGE following reduction (data not shown). Misfolded glycoproteins (25) and MHC class I protein molecules (32) are known to be retained in such aggregates in the ER and in the intermediate Golgi compartment. The delayed delivery of the C282Y mutant protein from the ER to the middle Golgi is likely the cause of the accelerated degradation of a large fraction of the newly synthesized C282Y protein (33). These data agree with those of Feder *et al.* (19) that association of  $\beta_2$ M is necessary for transport of HFE protein to the cell surface. We suggest that the C282Y mutation, which prevents this association, results in a reduction in the amount of mutant protein delivered from ER to Golgi, and also prevents delivery to the cell surface because of a block in the transit of the protein from the middle to trans Golgi compartment.

Unlike the C282Y mutant protein, the H63D mutant protein associates with  $\beta_2$ M. In addition, its synthesis, intracellular transport, oligosaccharide processing, and cell surface expression in COS-7 cells are all similar to those of wild-type HFE protein. Despite its apparently normal behavior in all these functional criteria, the enrichment of the H63D alleles in HH patients over that predicted from its frequency in the general population (6, 8) suggests that it can contribute to HH, at least when present in combination with the C282Y allele. Although the H63D residue is a nonconserved residue in other MHC class I proteins and in the human Fc receptor, the H63D mutation is located near the area of the polypeptide binding groove of HLA molecules (17). We speculate that the H63D mutation in the HFE protein reduces the affinity for an iron sensor protein or an iron binding protein present inside the cell or on the cell surface.

Iron absorption is a tightly regulated process and depends on the body's demand for iron. Studies have shown that there is zonal specialization in the handling of iron in the small intestine, with the mature epithelial cells of the mid to upper villus being the site of dietary iron absorption, while the cells of the intestinal crypts are able to transport iron from the circulation via transferrin receptors (16). Therefore, it appears that it is the crypt cells that are responsive to the body's demands for iron and are the site of regulation of iron absorption, although the capability for iron absorption is only expressed after these cells migrate to and become mature enterocytes in the villus. The biochemical mechanisms by which the crypt cells respond to the body iron level may be a key question in HH, where the rate of iron absorption is inappropriately high for the body iron burden. Interestingly, it is the crypt cells that express HFE protein in the small intestine (15). This raises the possibility that HFE protein may serve as an iron sensor or interact with an iron binding protein in the crypt cells, resulting in the genetic programming for iron absorption that is realized when the crypt cells mature into villal enterocytes.

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