The Hemochromatosis Founder Mutation in HLA-H Disrupts \(\beta_2\)-Microglobulin Interaction and Cell Surface Expression*

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We recently reported the positional cloning of a candidate gene for hereditary hemochromatosis (HH), called HLA-H, which is a novel member of the major histocompatibility complex class I family. A mutation in this gene, cysteine 282 → tyrosine (C282Y), was found to be present in 83% of HH patient DNAs, while a second variant, histidine 63 → aspartate (H63D), was enriched in patients heterozygous for C282Y. The functional relevance of either mutation has not been described. Co-immunoprecipitation studies of cell lysates from human embryonic kidney cells transfected with wild-type or mutant HLA-H cDNA demonstrate that wild-type HLA-H binds \(\beta_2\)-microglobulin and that the C282Y mutation, but not the H63D mutation, completely abrogates this interaction. Immunofluorescence labeling and subcellular fractionations demonstrate that while the wild-type and H63D HLA-H proteins are expressed on the cell surface, the C282Y mutant protein is localized exclusively intracellularly. This report describes the first functional significance of the C282Y mutation by suggesting that an abnormality in protein trafficking and/or cell-surface expression of HLA-H leads to HH disease.

Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism and represents one of the most common inherited disorders in individuals of Northern European descent with an estimated carrier frequency between 1 in 8 and 1 in 10 (1, 2). In patients with HH, excessive iron deposition in a variety of organs leads to multi-organ dysfunction. Recently, we reported a mutation in a novel MHC class I-like gene, called HLA-H (3). Eighty-three percent of HH patients' DNAs were found to be homozygous for this mutation, which consists of a single base transition of G to A and results in a change of cysteine 282 → tyrosine (C282Y). Subsequent reports have confirmed the high frequency of this founder mutation in other HH patients (4–6), providing further support that HLA-H is the primary HH locus. A second missense mutation, histidine 63 → aspartate (H63D), was also reported that was enriched in heterozygotes with the C282Y mutation (eight of nine cases) (3). The specific role that either of these mutations in HLA-H play in the etiology of HH disease has not been elucidated.

The HLA-H protein is similar to MHC class I family molecules including HLA-A2, nonclassical class I molecules such as HLA-G, and the human neonatal Fc receptor (FcRn). All four of the invariant cysteine residues that form disulfide bridges in the \(\alpha_2\) and \(\alpha_3\) domains of MHC class I family members are present in the HLA-H protein. One of these conserved cysteine residues is altered in the C282Y mutation. The integrity of the conserved disulfide linkages has been suggested to be critical for proper maintenance of the secondary and tertiary structure of the protein allowing interactions with accessory molecules such as \(\beta_2\)-microglobulin (7). Importantly, the functional significance of an interaction between \(\beta_2\)-microglobulin and an unknown class I-like molecule in HH disease was suggested by \(\beta_2\)-microglobulin-deficient mice; these mice display a progressive hepatic iron overload similar to that observed in human HH (8–10). Other studies have demonstrated that mutation of cysteine 203 in the \(\alpha_3\) domain of the mouse MHC class I family member H-2L \(\delta\) prevented intracellular transport of the molecule from the endoplasmic reticulum to the plasma membrane (11).

As a step toward understanding the role of HLA-H in HH disease, we examined the effects of the C282Y and H63D mutations on HLA-H cellular processing. In this report we demonstrate that wild-type HLA-H binds \(\beta_2\)-microglobulin and that the C282Y mutation completely abrogates this interaction and disrupts intracellular protein trafficking. The data provide support for the hypothesis that the C282Y mutation results in intracellular sequestration of the HLA-H protein, which leads to HH disease.

EXPERIMENTAL PROCEDURES

Cloning of HLA-H Wild-type and Mutant cDNAs—The HLA-H cDNA was fused to the FLAG octapeptide sequence (Eastman Kodak Co.) to utilize specific available antibodies for detection of the HLA-H protein. We designed a 3′ PCR primer oligonucleotide that altered the natural stop codon of the cDNA without an amino acid change and added the FLAG sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) with a termination codon and a unique NotI site for cloning into pcDNA 3.1 expression vectors (Invitrogen). The 3′ primer sequence was: 5′-ATG-CCG-TAG-CGG-CGG-CCT-ATC-ACT-TGT-CAT-GGT-CGT-TCT-TGT-AGT-CCT-CAC-GTT-CAG-CTA-AGA-CGT-AG-3′. The 5′ primer sequence was 5′-CAT-GCG-ATC-CAC-CAT-GGG-CCC-GGC-AGC-CAG-CGC-GGC-GCC-GTC-TCT-C-3′, which serves to add a unique BamHI site at the 5′ end and maintains the natural initiation codon.

To isolate the H63D mutant cDNA we started with first strand cDNA from a patient known to have that mutation and utilized the primer pair from above to amplify the desired clone. For the C282Y mutation we utilized a standard PCR mutagenesis approach: two overlapping fragments with the appropriate G to A base change were produced in a first-round PCR reaction, and the fragments gel-purified and combined with the 5′ and 3′ primers used above to yield the appropriately mutated cDNA with the FLAG sequence attached to the 3′ end.
Polyclonal Antipeptide Antibodies—Three peptides were synthesized (Multiple Peptide Systems) corresponding to amino acid sequences of the HLA-H protein. Two were to the predicted extracellular region of the molecule: peptide EX1 comprising amino acids 164–177 of the α domain (CFRAWPITLWEHRK) and peptide EX2 comprising amino acids 246–260 of the α domain (CKDKQFMDAKEFEPKD). The third, CT1, was from the putative cytoplasmic tail comprising amino acids 326–343 (CRQSGRGMAGHVLAEK). For each, an NH₂-terminal cysteine residue was incorporated to enable directed coupling to keyhole limpet hemocyanin. Rabbit antisera were produced and the resulting polyclonal antisera named utilizing the peptide nomenclature.

Immunoprecipitations and Western Blotting—Cells were lysed in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS) plus 0.5% Nonidet P-40 and the HLA-H or β₂-microglobulin proteins immunoprecipitated from 1 mg of total cell protein with 50 μg of FLAG M2 monoclonal antibodies (Eastman Kodak Co.) or 6 μg of β₂-microglobulin monoclonal antibody B1G6 (ImmunoTech) followed by addition of protein G-Sepharose (Pharmac Biotech Inc.). After washing three times with TBS containing 0.5% Tween 20, the antibody-antigen complexes were dissociated by heating at 100 °C for 4 min in standard Laemmli sample buffer and the material separated on a 4–20% Tris-glycine gradient polyacrylamide gel (Novex). Gels were electroblotted onto PVDF membranes (Novex), incubated with either 2 μg/ml FLAG M2 antibody, 25 μg/ml polyclonal β₂-microglobulin antibody (Boehringer Mannheim), or 2 μg/ml CT1 HLA-H antibody and the immune complexes detected by ECL (Amersham Corp.) utilizing horseradish peroxidase-linked sheep anti-mouse antibodies or horseradish peroxidase-linked donkey anti-rabbit antibodies as appropriate.

Subcellular Fractionation—Membrane fractionation was performed as described previously (12). Three membrane fractions (“light,” 20%/50% sucrose interface) were obtained from approximately 1×10⁶ cells by sucrose step-gradient centrifugation of postnuclear membrane fractions. Membranes from each step-gradient interface representing membranes from 1×10⁶ cells were mixed with Laemmli sample buffer and proteins separated and blotted as before. HLA-H proteins were detected with a 1:1 mixture of EX1 and EX2 antibodies at 5 μg/ml. For detection of subcellular markers, protein blots were probed with mouse monoclonal antibodies to Na⁺/K⁺-ATPase (plasma membrane), calnexin (endoplasmic reticulum, ER), and a rabbit polyclonal antibody to β-COP (Golgi) all obtained from Affinity Bioreagents. Specific bands were quantitated using a scanning densitometer (Molecular Dynamics).

Immunofluorescence Microscopy—Parental and transfected 293 stably cell lines were seeded on rat-tail collagen (Biomedical Technologies)-coated glass coverslips and grown overnight in standard medium. Cells were fixed in 3.5% paraformaldehyde, stained with a 1:1 mixture of affinity-purified antibodies EX1 and EX2 at 50 μg/ml or FLAG-M2 antibodies at 25 μg/ml and immune complexes detected with fluorescein isothiocyanate-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibodies, respectively (Zymed Laboratories Inc.). To detect intracellular antigen, cells were first fixed and then permeabilized with 0.5% saponin in phosphate-buffered saline for 3 min at room temperature prior to exposure to the primary antibodies. For peptide competition experiments, peptides (EX1, EX2, and M2) were incubated at a 100-fold molar excess with their respective antibodies for 1 h at room temperature prior to application to the cover slips.

RESULTS AND DISCUSSION

We first sought to demonstrate an interaction of the HLA-H protein with β₂-microglobulin and to examine the effects of the C282Y and H63D mutations on that interaction. Human embryonic kidney cells (293 cells) were transfected with vectors containing the wild-type HLA-H cDNA or the cDNA with either the C282Y or H63D mutation (Fig. 1A). The results with the H63D mutant were similar to the wild-type HLA-H protein; β₂-microglobulin was co-immunoprecipitated along with that mutant protein (Fig. 1A, left panel, compare H63D and Wild type lanes). It is of interest to note that the wild-type or H63D HLA-H proteins detected in the right panel appeared to migrate as a doublet of 49 and 46 kDa in lighter exposures, whereas the C282Y appeared as only a single band of approximately 46 kDa. This failure to detect β₂-microglobulin was not due to lack of HLA-H protein in the mutant cell lines, since stripping the blots and immunodetection with rabbit polyclonal antibodies directed to the COOH-terminal 17 amino acids of HLA-H (CT1 antibodies) demonstrated that the amount of HLA-H protein in the three cell lines was similar (Fig. 1A, right panel). The results with the H63D mutant were similar to the wild-type HLA-H protein; β₂-microglobulin was co-immunoprecipitated along with that mutant protein (Fig. 1A, left panel, compare H63D and Wild type lanes).

C282Y mutation (Fig. 1A, left panel). This failure to detect β₂-microglobulin was not due to lack of HLA-H protein in the mutant cell lines, since stripping the blots and immunodetection with rabbit polyclonal antibodies directed to the COOH-terminal 17 amino acids of HLA-H (CT1 antibodies) demonstrated that the amount of HLA-H protein in the three cell lines was similar (Fig. 1A, right panel). The results with the H63D mutant were similar to the wild-type HLA-H protein; β₂-microglobulin was co-immunoprecipitated along with that mutant protein (Fig. 1A, left panel, compare H63D and Wild type lanes). It is of interest to note that the wild-type or H63D HLA-H proteins detected in the right panel appeared to migrate as a doublet of 49 and 46 kDa in lighter exposures, whereas the C282Y appeared as only a single band of approximately 46 kDa.

The β₂-microglobulin/HLA-H interaction results were corroborated by performing the inverse experiment in which cell lysates were initially immunoprecipitated with β₂-microglobulin antibodies followed by Western blotting with antibodies directed toward the COOH-terminal sequence of HLA-H (CT1...
antibodies). In this experiment, the β2-microglobulin antibodies co-immunoprecipitated HLA-H protein from the wild-type and H63D mutant expressor cell lines, but failed to do so in the C282Y mutant expressor cell line (Fig. 1B, left panel). Stripping the blots and reprobing with β2-microglobulin antibodies demonstrated that similar amounts of β2-microglobulin protein were immunoprecipitated from each cell line (Fig. 1B, right panel). These results further confirm an interaction between wild-type HLA-H protein and β2-microglobulin and demonstrate that the C282Y, but not the H63D, mutation disrupts this association.

Previous reports have suggested that association of the MHC class I heavy chain with β2-microglobulin is critical for cell-surface expression (14, 15). Because of the failure of the HLA-H protein containing the C282Y mutation to interact with β2-microglobulin, we next investigated whether this mutation would also affect cell-surface presentation of the HLA-H protein. Parental 293 cell lines and those expressing the wild-type HLA-H protein or the C282Y mutant were examined for cell-surface protein expression by immunostaining with rabbit polyclonal antibodies specific to sequences residing in the predicted external domain of the HLA-H protein (EX1 and EX2 antibodies) followed by detection with immunofluorescence. Parental 293 cells displayed no detectable surface labeling by these antibodies (Fig. 2A), consistent with the undetectable levels of HLA-H protein observed in the Western blotting experiments (Fig. 1A, right panel). Wild-type HLA-H-expressing cells demonstrated a distinct pattern of surface labeling as evidenced by a punctate pattern of labeling that was much more intense at the edges of the cells (Fig. 2B). By contrast, cells expressing the C282Y mutation displayed no surface labeling and were indistinguishable from the parental controls (Fig. 2, compare C and A). The specificity of the antibody labeling was demonstrated by preincubating the EX1 and EX2 antibodies with their respective peptides; in these experiments the punctate surface labeling observed in the wild-type HLA-H expressor cells was completely abolished (data not shown).

We examined the possibility that the C282Y mutant protein was expressed in the transfected cells but remained intracellularly localized. Immunostaining was performed following treatment of the cells with saponin to permeabilize them. Staining of these cells for the FLAG-tagged C282Y mutant HLA-H protein demonstrated strong perinuclear fluorescence, which was absent in the parental control cells (Fig. 2, compare D and F). Permeabilized wild-type HLA-H protein expressor cells showed similar intracellular staining with the FLAG-M2 antibody, suggesting that not all of the wild-type protein in these transfected cells reaches the cell surface (E). Experiments utilizing the EX1, EX2, or CT1 antibodies yielded the same results (data not shown). These results clearly demonstrate that the C282Y mutation specifically disrupts cell-surface presentation of the HLA-H protein.

To examine the distribution of wild-type and mutant HLA-H proteins within the cell in more detail, we performed subcellular fractionations on stepwise sucrose gradients to separate the various membrane components. Three separate postnuclear membrane fractions were obtained; the 20/35% interface contained the lightest density membranes (L); dense membranes (D) partitioned at the 40/50% interface, whereas the 35/40% medium-density (M) interface contained a mixture of light and dense membrane-derived components. We initially characterized the efficacy of our subcellular membrane fractionation scheme by assaying these fractions for marker proteins. Antibodies to Na+/K+ ATPase were utilized as markers for plasma membrane, β-coatomer protein (β-COP) for Golgi, and calnexin for ER membrane identification. Samples representing membranes from equal numbers of cells from each interface were analyzed by Western blotting and quantitated on a Molecular Dynamics scanning densitometer. Plasma membranes were found primarily in the light-density interface and to a lesser extent in the medium-density layer: L, 90%; M, 10%; D, 0%. Golgi membranes were distributed nearly equally throughout the three interfaces: L, 30%; M, 40%; D, 30%. ER membranes were found mostly in the dense membrane interface: L, 0%; M, 20%; D, 80%. The fractionations from each of the three cell lines gave equivalent results.

We determined the specific distribution of HLA-H proteins in the sucrose gradient interfaces by Western blotting and probing with HLA-H antibodies. As with the co-immunoprecipitation results (Fig. 1), the immunostaining suggested that the wild-type HLA-H protein migrated as a doublet of the 49 and 46 kDa forms (Fig. 3, left panel). The slower migrating 49-kDa form was found principally in those fractions containing plasma membranes, whereas the lower molecular mass 46-kDa form was distributed in a pattern similar to that of the Golgi marker, β-COP. By contrast, the C282Y mutant HLA-H protein consisted only of the faster migrating 46-kDa species. Like the wild-type 46-kDa protein, the mutant 46-kDa protein was distributed in a pattern that most closely resembled that of the Golgi marker protein, suggesting the possibility of incomplete posttranslational processing or modification (Fig. 3, middle panel). The H63D mutant proteins migrated in a pattern resembling that of the wild-type protein, implying that this mutation had little or no effect on intracellular HLA-H protein.
trafficking (Fig. 3, right panel). In other studies, no HLA-H protein was detected in the cytosolic fractions of any of the wild-type or mutant cell lines, suggesting that neither the C282Y nor the H63D mutation cause a redistribution of the protein to the cytoplasm (data not shown).

Taken together these results demonstrate that the C282Y mutation prevents the HLA-H molecule from interacting with β2-microglobulin and eliminates cell-surface presentation. Cysteine 282 is one of four cysteine residues that are invariant in both classical and nonclassical MHC class I molecules and forms a critical disulfide bridge in the α3-immunoglobulin-like domain (7). Thus, the integrity of this structure is critical to the formation of the heterodimer of β2-microglobulin and HLA-H and also for proper intracellular processing of the protein. Class I MHC molecules are noncovalently linked heterodimers between an α heavy chain and β2-microglobulin (light chain) (7). The role of the β2-microglobulin-heavy chain interaction is to facilitate and stabilize the folding of the heavy chain during biosynthesis through interactions with the α1–α2 platform and the α3 domain (16) (7). Previous work demonstrated that mutating the reciprocal cysteine residue (cysteine 203) in mouse H-2Ld alters the subcellular distribution of the protein. Membranes from 293 cells stably expressing the wild-type HLA-H or the C282Y or H63D mutant proteins were fractionated by isopycnic stepwise sucrose gradient centrifugation as described under “Experimental Procedures.” Membranes from each interface were then resolved on SDS-PAGE, transferred to PVDF membrane, and the HLA-H protein detected with a 1:1 mixture of EX1 and EX2 HLA-H antibodies followed by ECL. L, 20/35% sucrose light-density interface; M, 35/40% sucrose medium-density interface; D, 40/50% sucrose high-density interface; WT, wild-type HLA-H expressor cell line; C282Y, C282Y HLA-H mutant expressor cell line; H63D, H63D HLA-H mutant expressor cell line.

FIG. 3. The C282Y mutation in HLA-H alters the subcellular distribution of the protein. Membranes from 293 cells stably expressing the wild-type HLA-H or the C282Y or H63D mutant proteins were fractionated by isopycnic stepwise sucrose gradient centrifugation as described under “Experimental Procedures.” Membranes from each interface were then resolved on SDS-PAGE, transferred to PVDF membrane, and the HLA-H protein detected with a 1:1 mixture of EX1 and EX2 HLA-H antibodies followed by ECL. L, 20/35% sucrose light-density interface; M, 35/40% sucrose medium-density interface; D, 40/50% sucrose high-density interface; WT, wild-type HLA-H expressor cell line; C282Y, C282Y HLA-H mutant expressor cell line; H63D, H63D HLA-H mutant expressor cell line.

...similar to the Golgi marker protein β-COP in subcellular fractions, but because of the limited resolution of the step-gradient, we cannot rule out that some protein may also be in the ER. The perinuclear pattern of staining noted in the immunofluorescence studies does not definitively resolve this. More detailed studies will be necessary to ascertain the specific point at which intracellular transport of the C282Y mutant is disrupted.

In contrast to our results with the C282Y mutation, we found no detectable changes in the β2-microglobulin interaction or intracellular processing of the H63D mutant form of HLA-H, which is enriched in C282Y heterozygous patients (3). Other studies have demonstrated alterations in intracellular transport of class I molecules by mutations in the peptide-binding groove of HLA-A (22). The H63D mutation is localized in the α2 domain between the third and fourth β strands of the external peptide-binding region. It is possible that the effect of this mutation is to alter the affinity of the HLA-H protein for an as yet unidentified ligand or to alter the manner that the mutant protein interacts with other proteins in the cell membrane. Alternatively, this mutation may represent a common polymorphism with little or no effect on the biological functioning of the protein. The definitive answer to this question will await further investigation as we elucidate how the HLA-H molecule regulates iron metabolism in the body.

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REFERENCES

1. Dadone, M. M., Kushner, J. P., Edwards, C. Q., Bishop, D. T., and Skolnick, M. H. (1982) Am. J. Clin. Pathol. 76, 196–207

2. Edwards, C. Q., Griffen, L. M., Goldgar, D., Drummond, C., Skolnick, M. H., and Kushner, J. P. (1988) N. Engl. J. Med. 318, 1355–1362

3. Feder, J. N., Gniere, A., Thomas, W., Tsuchihashi, Z., Ruddy, D. A., Basava, A., Dormishian, F., Domingo, R., Ellis, M. C., Fullan, A., Hinton, L. M., Jones, N. L., Kimmel, B. E., Kronaul, G. S., Lauer, P., Lee, V. K., Loeb, D. B., Mapa, P. A., McClelland, E. E., Meyer, N. C., Mintier, G. A., Moeller, N. N., Moore, T., Morikang, E., Frass, C. E., Quintana, L., Starnes, S. M., Schatzman, R. C., Brunke, K. J., Drayna, D. T., Rice, N. J., Bacon, B. R., and Wolff, R. K. (1996) Nat. Genet. 14, 499–508

4. Jaswinska, E. C., Cullen, L. M., Busfield, P., Pyper, W. R., Webb, S. I., Powell, L. W., Morris, C. P., and Walsh, T. P. (1990) Nat. Genet. 14, 250–251

5. Jouanolle, A. M., Gandon, G., Jenequel, P., Blayau, M., Campion, M. L., Yangping, J., Masser, J., Fregout, P., Chauvel, B., Bous, C., Garnier, N., Gicquel, I., Gall, J.-Y., and David, V. (1996) Nat. Genet. 14, 251–252

6. Beutler, E., Gelbart, T., West, C., Lee, P., Adams, M., Blackstone, R., Pockros, P., Kosty, M., Vendetti, C. P., Phatak, P. D., Seese, N. K., Chorney, K. N., Elleof, A. T. E., Gerhard, G. S., and Chorney, M. (1996) Blood Cells Mol. Dis. 22, 187–194

7. Bjorkman, P. J., and Parham, P. (1990) Annu. Rev. Biochem. 58, 235–288

8. de Souza, M., Reimao, R., Lacerda, R., Hugo, P., Kaufmann, S. H. E., and Porto, G. (1994) Immunol. Lett. 39, 105–111

9. Rothenberg, B. E., and Voland, J. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1529–1534

10. Santos, M., Schulich, M. W., Ramakers, L. H., Marx, J. J., de Souza, M., and Wolff, R. K. (1996) Nat. Genet. 15, 250–251

11. Miyazaki, J., Appella, E., and Ozato, K. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 757–761

12. Privalsky, M. L., and Bishop, J. M. (1984) Virology 135, 35–368

13. Ford, C. F., Suominen, I., and Glattz, C. E. (1991) Protein Expression Purif. 2, 95–107

14. Arce-Gomez, B., Jones, E. A., Barnstable, C. J., Solomon, E., and Bodmer, W. F. (1978) Tissue Antigens 11, 96–112

15. Seong, R. H., Claytonberger, C. A., Krenskey, A. M., and Parnes, J. R. (1988) J. Exp. Med. 167, 288–299

16. Hansen, T. H., Myers, N. B., and Lee, D. R. (1988) J. Immunol. 140, 3522–3527

17. Ploegh, H. L., Cannon, L. E., and Strominger, J. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2273–2277

18. Krangel, M. S., Orr, H. T., and Strominger, J. L. (1979) Cell 18, 979–991

19. Owen, M. J., Kissner_AMD, A.-M., and Lodish, H. L. (1980) J. Biol. Chem. 255, 1675–1684

20. Williams, D. B., and Watts, T. H. (1995) Curr. Opin. Immunol. 7, 77–84

21. Hsu, V. W., Yuan, L. C., Nuchtern, J. G., Lippincott-Schwartz, J., Hammerling, G. J., and Klaussner, R. D. (1991) Nature 352, 441–444

22. Salter, R. D. (1994) Immunogenetics 39, 266–271