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Study of the immunological role of HFE mutations

Instituto de Ciências Biomédicas de Abel Salazar
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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1 - GENERAL INTRODUCTION – PART I

The MHC-I antigen presentation pathway

Following infection, some pathogens assault the host cells establishing themselves in the sheltered intracellular milieu. This presents the immune system a continuous challenge trying to spot and destroy the invading species. However, centuries of a coevolutionary history have shaped an increasingly fine-tuned host immune response, equipped and eager to make the pathogen's life a rather complicated venture. In this hide-and-seek relationship, the pathogens fight to gain control of one particular piece of the host's defensive arsenal: the class I major histocompatibility complex (MHC-I) antigen presentation pathway. Viruses represent probably the most prominent example of this effort to overcome the host's cellular defences.

From fish to humans, all nucleated cells constitutively process and present short peptides derived from endogenously synthesized proteins. These peptides snug in the binding groove of MHC-I molecules that, after moving to the cell surface, enable CD8⁺ T-lymphocytes to spot intracellular pathogens such as viruses.

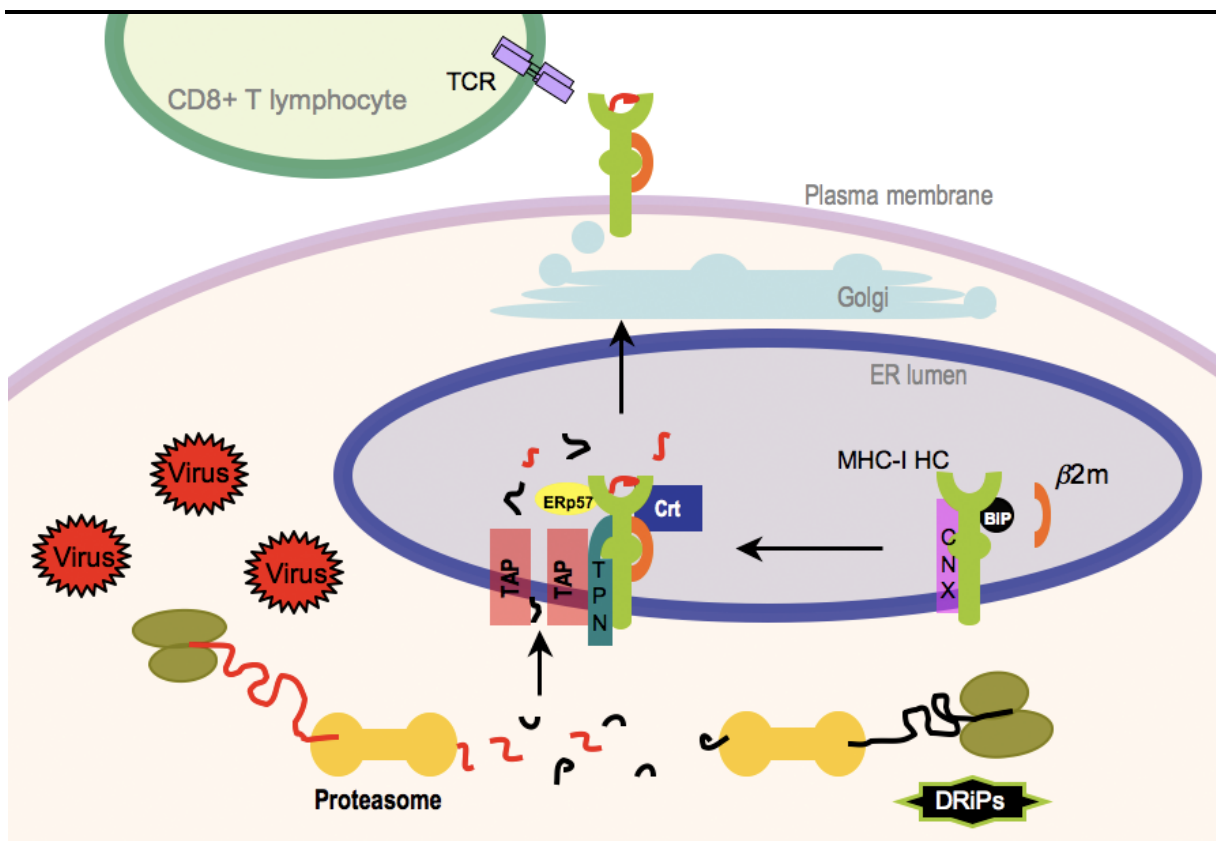


Figure 1 – The MHC-I antigen processing and presentation pathway. In the ER lumen, newly synthesized MHC-I heavy chains (HC) assemble with β2m. The interaction with the peptide loading complex (calreticulin, Erp57, tapasin and TAP) promotes peptide loading onto MHC-I molecules. The peptides are derived from endogenous antigens (including viral-derived proteins – represented in red), which are degraded by the proteasome into short peptides and transported into the ER lumen by TAP. The peptide loaded MHC-I complexes proceed to the cell surface via the standard secretory pathway, for presentation to CD8⁺ T-lymphocytes. CNX – Calnexin; Crt - Calreticulin; TPN – Tapasin; DRiPs – Defective ribosomal products; TCR – T-cell receptor.

Designing a peptide loaded MHC-I protein

Although conceptually simple, the aim being to display a fingerprint of the intracellular content at the cell surface by means of small protein fragments, antigen presentation is a rather sophisticated process involving a number of crucial events: MHC-I folding and maturation, peptide generation and shuttle into the endoplasmic reticulum (ER), assembly of the peptide loading complex (PLC), trafficking of peptide loaded MHC-I to the cell surface and ultimately its inspection by CD8⁺ T-lymphocytes (Figure 1).

MHC-I antigen presentation is initiated in the ER where folding of MHC-I molecules takes place (see Chapter 1 – Part III for further details on protein folding in the ER). This is a complex process, which involves the participation of a variety of auxiliary factors, like heavy-chain binding protein (BiP - also termed GRP78), the lectin chaperones calnexin and calreticulin, and the oxidoreductase ERp57 (Paulsson and Wang 2003). Following translocation into the ER via the Sec61 protein complex, MHC-I heavy chains (HC) bind to the chaperones BiP and calnexin and associate non-covalently with the soluble β 2-microglobulin (β 2m) (Bouvier 2003; Dick 2004). Upon β 2m association, calnexin is replaced by calreticulin and the heterodimer is recruited to a multisubunit structure, collectively termed the PLC, containing, at least, tapasin, calreticulin, ERp57, and the transporter associated with antigen presentation (TAP) (Cresswell et al. 1999).

The newly generated peptide-receptive MHC-I molecules are recruited into the PLC where they are stabilized until peptide loading takes place. A heterodimer of two membrane spanning molecules, termed TAP1 and TAP2 translocates the peptides from the cytosol to the ER lumen, where loading onto MHC-I molecules takes place (Androlewicz et al. 1993; Neefjes et al. 1993a). This process favors the production of stable MHC-I/peptide complexes (Elliott and Williams 2005). The action of tapasin, another component of the PLC, is thought to play a decisive role in the formation of low off-rate MHC-I/peptide complexes. Two lines of evidence provide a rationalization for this: the first is that tapasin edits the peptide repertoire in order to assure optimal MHC-I peptide loading (Howarth et al. 2004); the second explanation suggests that tapasin facilitates the binding of a more diverse set of peptides by stabilizing the MHC-I peptide-receptive conformation, but does not function as a peptide editor to discriminate between low and high affinity peptides (Zarling et al. 2003). The definition of the precise mechanisms by which tapasin partakes on the assembly of low off-rate MHC-I/peptide complexes is still a matter of debate. Nevertheless, its crucial involvement in this process is supported by the observation that the lack of tapasin leads to a reduced stability of the MHC-I complexes at the cell surface (27–31). The hypothesis that tapasin may not act alone in peptide loading optimization was recently supported by the finding that protein disulfide isomerase (PDI), another component of the PLC, stabilizes a peptide-receptive conformation of MHC-I by regulating the oxidation state of the disulfide bond in the peptide binding groove (Park et al. 2006).

MHC-I molecules that fail to acquire high affinity peptides exit the ER from a site distinct from that used by high-affinity MHC-I/peptide complexes, and are subjected either to degradation or short term expression at the cell surface followed by endocytosis and degradation (Pentcheva and Edidin 2001). MHC-I molecules, once loaded with high-affinity peptides, dissociate from the PLC, leave the ER at specific exit sites, transit through the Golgi apparatus, and emerge at the cell surface (Spiliotis et al. 2000). Here, they are available for scrutiny by

circulating CD8⁺ T-lymphocytes that obtain access to a detailed snapshot of the intracellular environment. Eventually, if a strange (non-self) peptide is spotted, a cytotoxic immune response against the infected cell is initiated.

The source of the MHC-I peptides

Protein turnover is a common feature of all organisms. Sooner or later, all proteins are degraded as a way of controlling their properties and their relative levels. This constant supply of protein fragments might satisfy the entire antigen presentation pathway needs and, since all proteins will eventually face proteasomal degradation, it allows for a vast and truthful portfolio of the intracellular content to be displayed by MHC-I molecules. However, protein turnover is a rather slow process. The half-life of some proteins can exceed one week and, in average, 1-2 days are needed for a protein to start the recycling process (Goldberg and Dice 1974). Since viruses can generate their progeny in few hours after infection, a distinct mechanism must operate in the host cells to assure detection of pathogens with sufficient alacrity. In fact, a shortcut between translation and peptide generation guarantees the efficiency and promptness of antigen presentation: defective ribosomal products (DRiPs) are the major source of antigenic peptides for MHC-I presentation (Yewdell et al. 1996).

DRiPs consist of viral and cellular proteins that fail to acquire a native conformation due to errors in transcription, translation, folding, intracellular trafficking or assembly. The findings that blocking protein synthesis with cycloheximide reduces peptide translocation by TAP and MHC-I export from the ER to the same extent as proteasome inhibition have opened the door to the theory that the major substrates for peptide generation are derived from newly synthesized proteins (Reits et al. 2000; Schubert et al. 2000). Moreover, it was shown that inhibition of protein synthesis impairs MHC-I processing specifically by depleting the pool of rapidly degraded proteins ($t_{1/2}$ of ~10 min), such as DRiPs, and that MHC-I peptides are preferentially generated from these short-lived proteins (Qian et al. 2006).

Unlike protein turnover, degradation of DRiPs offers a prompt collection of peptides for MHC-I loading. This close link between translation and antigen presentation allows a proficient immune response in situations where time is crucial like those following acute virus infections, in which efforts rush to diminish viral replication and transmission.

Hijacking MHC-I: viruses take control

The fact that the main source of peptides for MHC-I scrutiny are DRiPs enables the immune surveillance mechanisms to detect pathogens, which employ the host cell for protein synthesis (Reits et al. 2000; Yewdell et al. 2003). After infection, viruses reprogram the host's machinery to synthesize viral proteins, which are produced at very high levels. As a result, the complexity of the MHC-I peptide repertoire is biased toward peptides of viral origin (Yewdell et al. 2003). To deal with this apparent limitation, several viruses have evolved sophisticated strategies to prevent the generation or MHC-I presentation of antigenic peptides, causing the immune system subversion, assuring a lifelong persistence and repeated reactivation within the host. Essentially, all MHC-I antigen presentation pathway events are targeted.

Due to its pivotal role on the MHC-I antigen presentation, one of the major aims of viruses is the PLC. ICP47, a herpes simplex virus (HSV) protein, prevents peptide binding and translocation by TAP (Tomazin et al. 1996). This blocks MHC-I peptide loading and, as a consequence, causes its ER retention and proteasomal degradation (Hughes et al. 1997). A similar mechanism is employed by human cytomegalovirus (HCMV) US6 protein that blocks ATP binding to TAP inhibiting peptide translocation (Ahn et al. 1997; Hewitt et al. 2001). The murine γ -herpesvirus-68 also aims components of the PLC to escape immune detection. Its K3 derived protein (mK3) targets both TAP and tapasin for degradation (Boname et al. 2005). Moreover, mK3 protein leads to polyubiquitination and proteasomal degradation of newly synthesized MHC-I HCs (Boname and Stevenson 2001).

MHC-I itself is a preferential target for a number of different viruses attempting to evade immune recognition. HCMV proteins US2 and US11 provide the best characterized examples of viral-mediated degradation of MHC-I molecules. Like mK3, US2 and US11 induce premature proteasomal degradation of nascent class I HCs (Wiertz et al. 1996a; Wiertz et al. 1996b). Yet, the strategy employed differs from that of mK3. US2 and US11 are located in the ER lumen and do not have ubiquitin ligase activity. Instead, they bind to MHC-I molecules promoting their ER-to-cytosol dislocation where degradation occurs (see chapter 1 – Part III for further details on the ubiquitin-proteasome system and US2/US11 mediated MHC-I dislocation) (Gewurz et al. 2001).

Several other examples of immune evasion strategies employed by viruses exist. From the inhibition of proteolysis and peptide generation (HCMV E protein pp65 and human immunodeficiency virus (HIV) Tat) to internalization of MHC-I molecules from the cell surface (Kaposi sarcoma-associated herpes virus (KSHV) K3 and K5 and HIV Nef), viruses have developed sophisticated machinery designed to target virtually every step of the MHC-I antigen presentation pathway (Ambagala et al. 2005). However, in this historical narrative the converse is also true: the evolutionary pressure applied to the host might have led to the development of mechanisms that alleviate some of the suppressive effects of its parasites. This is of particular significance for host/pathogens that have a long natural history in common (e.g. infections by herpes virus). In such cases, a balance whereby the pathogen manipulates the immune system causing only minimal illness is achieved promoting the survival of both species.

HFE

HFE is a member of a rather large family of proteins sharing common structural features, which are encoded by a group of genes located on the MHC region in chromosome 6. The protein is a 343 residue type I transmembrane glycoprotein homologous to MHC-I molecules (such as HLA-A2, with which it shares 37% sequence identity) (Lebron et al. 1998). The extracellular portion of HFE consists of $\alpha 1$ and $\alpha 2$ domains and an immunoglobulin-like $\alpha 3$ domain. However, one significant structural difference exists between HFE and MHC-I: the putative peptide-binding groove of HFE is too narrow to accommodate a short peptide. The surface area of the groove formed by $\alpha 1$ and $\alpha 2$ domains is approximately 415 Å² compared to approximately 760 Å² in MHC-I molecules. Given this structural limitation and despite its homology with MHC-I, HFE does not present antigens to T-lymphocytes (Lebron et al. 1998) (see Chapter 1 – Part I for further details on MHC-I antigen presentation).

As for most transmembrane proteins, HFE folding occurs in the ER (see Chapter 1 – Part III for further details on protein folding), where the $\alpha 3$ domain in HFE interacts with the class I light chain $\beta 2m$ (Lebron et al. 1998). The heterodimer is then capable of leaving the ER, traveling through the standard secretory pathway toward the cell surface (Figure 2).

HFE is predominantly found in the duodenum, liver, pancreas, placenta, kidney, macrophages and ovary, while in colon, leukocytes, brain, and lung it is present in lower concentrations (Feder et al. 1996).

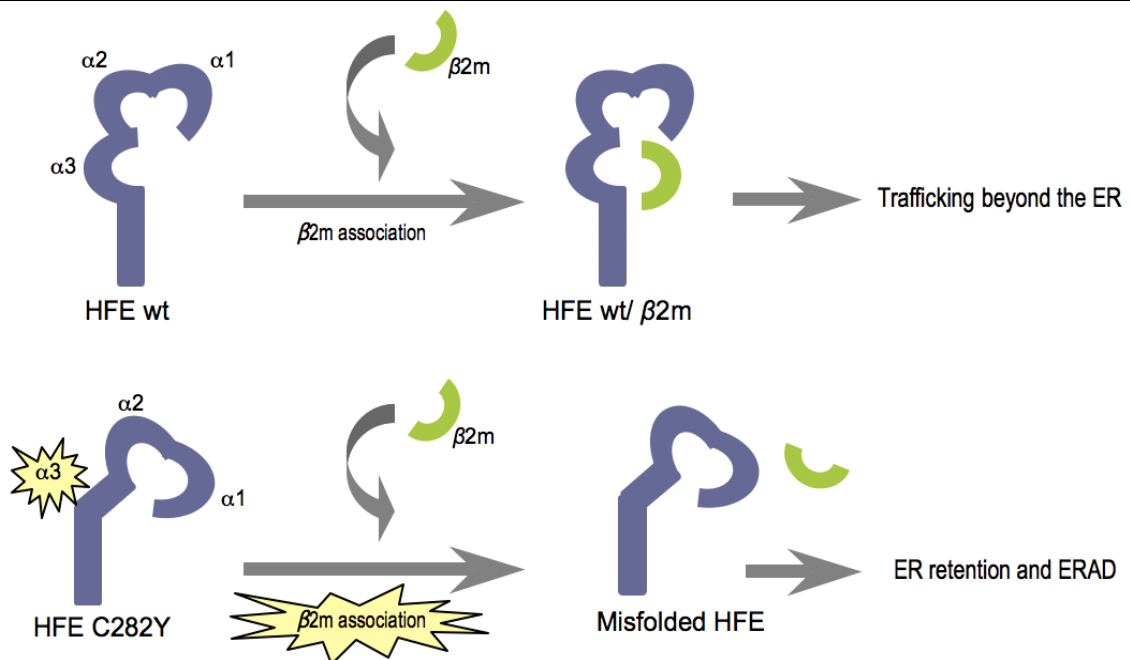


Figure 2 – Impact of the C282Y mutation on the HFE/ $\beta 2m$ association. In the ER lumen, HFE wt proteins assemble with $\beta 2m$ and exit the ER through the standard secretory pathway. The C282Y mutation abrogates the formation of the $\alpha 3$ domain impeding the association of HFE with $\beta 2m$. The resulting protein is retained in the ER and subjected to degradation. *The C282Y mutation*

In the C282Y HFE mutation a single-base transition leads to the substitution of tyrosine for cysteine at position 282. This provokes the disruption of the $\alpha 3$ domain intrachain disulfide bond, which is a mandatory prerequisite for $\beta 2m$ association, intracellular transport and expression of the HFE protein at the cell surface. As a result, the C282Y mutant protein does not fold correctly and fails to bypass the ER quality control mechanisms being trapped in the ER, from where it is subject to premature proteasomal degradation (Feder et al. 1997; Waheed et al. 1997) (Figure 2).

Another common HFE mutation, the H63D mutation, whose pathogenic significance is still uncertain, involves the substitution of a histidine by an aspartate at position 63. This mutation occurs in the $\alpha 1$ domain of the protein and does not perturb $\beta 2m$ association or cell surface expression of HFE protein (Feder et al. 1996).

HFE and Hereditary Hemochromatosis

Hereditary Hemochromatosis (HH) is the most common inherited disease in Caucasians. In these patients, intestinal iron absorption and iron export from macrophages are abnormally elevated resulting in progressive tissue iron overload which leads to irreversible organ damage if not treated timely (Pietrangelo 2006). The pancreas, heart, skin, joints and particularly the liver are the main deposition sites where the iron surplus leads to organ failure through the production of oxidant species. Cirrhosis, hepatocellular carcinoma, cardiomyopathy, diabetes, hypogonadotropic hypogonadism and arthritis are some of the pathologies associated with chronic iron overload (Niederau et al. 1994). Nevertheless, if started before the onset of irreversible organ damage, therapeutic phlebotomy - the mainstay of the treatment of HH - offers these patients a life expectancy similar to that of normal population.

The HFE C282Y mutation and HH

Inasmuch as the MHC-I-like proteins are typically involved in immunological processes, such as MHC-I antigen presentation to CD8+ T-lymphocytes, it would seem unlikely that one of its family members is involved in the iron metabolism. However, a number of findings eventually related HFE to HH. Nevertheless, the first clues about the identity of the gene implicated in HH were provided some time before the discovery of HFE. Back in 1994 it was found that the $\beta 2m$ knockout mice develops iron overload with a tissue distribution indistinguishable from that observed in HH (de Sousa et al. 1994). This raised the possibility that the gene product defective in HH might have a structure similar to MHC-I molecules and an analogous requirement for $\beta 2m$ association for proper folding and function. In fact, the mapping and cloning of the human HFE revealed its sequence homology to MHC-I molecules, in particular to HLA-A2 (Feder et al. 1996). Furthermore, the predicted HFE structure revealed that $\beta 2m$ association was a pre-requisite for the correct protein maturation (Lebron et al. 1998) and its involvement on iron metabolism was consistent with the iron overload found in the $\beta 2m$ knockout mouse (Santos et al. 1996; Feder et al. 1997).

The finding that over 83% of the cases of HH are associated with the C282Y mutation has established HFE as a key partaker in the regulation of iron homeostasis and in the progression of HH (Feder et al. 1996). In

agreement with a role in iron homeostasis and with the pathogenesis of the disease, high levels of HFE mRNA were found in tissues involved in iron mobilization and storage, like the duodenum, placenta, macrophages and the liver (Parkkila et al. 1997b). Adding to the evidence of an implication in iron metabolism, shortly after its discovery HFE was found to associate with the transferrin receptor (TfR) (Parkkila et al. 1997a) and to decrease its affinity for iron-bound transferrin by 5 to 10-fold (Feder et al. 1998). The C282Y mutation disrupts this association, thus preventing HFE's inhibitory role on iron uptake (Feder et al. 1998).

Besides the impairment on the iron uptake, HH is also characterized by defects on the iron export machinery and poor iron storage in reticuloendothelial cells is a major feature of the disease. In fact, it was found that HFE blocks cellular iron export from macrophages and that this function is lost in HH because of the C282Y mutation (Drakesmith et al. 2002).

The HFE's role in the regulation of cellular iron stores and, more significantly, the impact of the C282Y mutation on this task definitely implicated this particular mutation on the physiopathology of HH.

Immunological abnormalities of HH patients

New aspects of HFE's biology are emerging suggesting that its function might go beyond the exclusive participation on iron homeostasis toward a putative function in other unrelated processes. For instance, studies on immunological aspects of HH patients have revealed concealed clues of an unsuspected role of HFE in the immune system.

Analysis of the T-lymphocyte numbers showed that in comparison to an healthy control population, HH patients exhibit higher CD4⁺/CD8⁺ ratios (Reimao et al. 1991). This was found to result from abnormally lower CD8⁺ cells and not to higher CD4⁺ numbers (Porto et al. 1994). Moreover, a correlation was established between this parameter and the clinical expression of HH: a lower CD8⁺ T-lymphocyte population is associated with a more severe form of the disease (Porto et al. 1997). In addition to the T-lymphocyte population's discrepancies, functional studies revealed that, in comparison to healthy controls, CD8⁺ T-lymphocytes from HH patients exhibit a diminished cytotoxic activity (Arosa et al. 1997) and decreased CD8-p56lck activity (Arosa et al. 1994). This abnormality was not corrected by the phlebotomy treatments suggesting that it may be linked to aspects associated with HH other than the iron-mediated toxicity. One candidate might be HFE itself, particularly the impact of the C282Y mutation on a putative iron-independent function.

Even though all these findings strongly suggested an extended role related to the immune system, evidence for a direct involvement of HFE on a classical immunological process was described only recently. Using transgenic mice, it was observed a direct cytolytic recognition of human HFE by mouse T cell receptors (TCR), which occurs independently of HFE-bound peptides (Rohrlich et al. 2005). The physiological significance of this finding and its contribution to the disease progression in the presence of the C282Y mutation remains to be clarified.

The virus connection: hints for an extended role of HFE

Several examples of associations between viral-diseases and HH have been described in the past years. Most of these studies investigated the correlation between the frequency of HFE mutations and the susceptibility to infection. However, two independent studies have provided mechanistic evidence for a direct effect of HCMV and HIV-1 on proper HFE expression. Upon HCMV infection, US2 protein targets HFE for proteasomal degradation interfering with its expression on the cell surface (Ben-Arieh et al. 2001). Similarly, in macrophages, Nef (an HIV-1 protein important for the progression to AIDS) reroutes HFE to a perinuclear compartment causing a 90% cell surface expression reduction (Drakesmith et al. 2005). The authors claim that by targeting HFE, the virus might gain access to an increased intracellular iron pool, thus favouring the replication process. Yet, some contradictory data indicate that this might not be the reason why HFE deserves virus attention. As mentioned previously, in macrophages HFE blocks iron export (Drakesmith et al. 2002). Accordingly, upon a viral infection, macrophages would undergo intracellular iron depletion due to virus-mediated degradation of HFE, arguing against the iron-enrichment theory being the final purpose of HFE's destruction. Something else seems to justify this unexpected interest in a protein thought to be involved exclusively in iron metabolism. A putative role in immunological processes would certainly place HFE under virus threat. In fact, the obsession of virus for escaping recognition by the immune system has led to the identification of several proteins aimed at by an intricate virus-derived machinery. MHC-I is amongst the preferred targets and its decisive role in denouncing invasion of pathogens to the immune system definitely supports it (see Chapter 1 – Part I for further details on viral immune evasion).

Protein folding and quality control in the Endoplasmic Reticulum

Before reaching their final destinations, proteins enter the secretory pathway through the ER, where folding takes place. Once in the ER, proteins may acquire a number of different conformations, each with a different free energy. Protein folding can basically be defined as the succession of structural modifications that any given protein suffers until the conformation with the lowest free energy is reached (Bryngelson et al. 1995).

Proteins targeted to the ER are driven by a stretch of 17 to 35 hydrophobic residues at the N-terminus, the signal peptide, and cross the ER membrane through the Sec61 protein complex (Walter and Johnson 1994; Lutcke 1995). In the ER lumen two classes of proteins shepherd the folding process: molecular chaperones and folding enzymes. Through transient associations with client proteins, they catalyze slow folding events, preventing protein aggregation by minimizing unproductive interactions between the polypeptide chains (Ellis and Hemmingsen 1989; Gething and Sambrook 1992). However, despite the abundance of chaperones and components of the folding machinery, protein misfolding and aggregation lie at the basis of several diseases (Rutishauser and Spiess 2002).

Since proteins in the cytosol and mitochondria remain within the compartment where they fold, they are under constant surveillance of the chaperones that guided their maturation. In contrast, upon ER exit, secretory proteins can no longer rely on any chaperone assistance. This has forced the development of a refined and rigorous ER quality control (QC) system that ensures that only correctly folded proteins leave the ER (Ellgaard and Helenius 2003).

ER quality control: the stamp of (dis)approval

ER retention of unfolded or misfolded proteins involves binding by one or more of the molecular chaperones that act as “folding sensors”. The most prominent and well studied elements of this ER folding components are the molecular chaperones BiP and glucose regulated protein 94 (GRP94); the thiol disulfide oxyreductases PDI and ERp57; and the lectin like chaperones calnexin and calreticulin (Ellgaard and Helenius 2003).

Molecular chaperones in the ER QC

Molecular chaperones, the most abundant proteins in the ER lumen, bind transiently to newly synthesized proteins. Besides assisting the folding process, they are active players of the QC machinery (Hurtley et al. 1989). Molecular chaperones such as BiP recognize hydrophobic regions of peptides that are usually buried inside native proteins. This way, BiP detects unfolded proteins supporting their folding in the ER, and retains terminally misfolded proteins in a soluble conformation. After they are released from BiP, misfolded proteins are delivered to ER-associated degradation (ERAD) (Ellgaard and Helenius 2003). For instance, among several other unfolded substrates that expose hydrophobic segments, BiP is known to bind to immature antibody subunits keeping them in the ER (Haas and Wabl 1983).

The oxyreductases task in the ER QC

A second arm of the QC machinery operates through a thiol-mediated retention mechanism. As for hydrophobic patches, exposed sulfhydryl groups provide a signal for ER retention. For instance, premature ER exit of unassembled subunits of IgM (Sitia et al. 1990) is mediated by PDI, ERp72 and possibly many more oxidoreductases which form disulfide bonds with the single cysteine in the tailpiece of the IgM heavy chains until the mature IgM pentamer is formed by interchain disulfide bonds (Reddy et al. 1996).

The calnexin/calreticulin cycle

The third component of the ER QC machinery involves the recognition of the glycan moiety of client proteins that are subjected to the concerted action of a series of enzymes and molecular chaperones termed the calnexin/calreticulin cycle (Helenius and Aebi 2004). Core oligosaccharides of the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ structure are co-translationally attached to asparagine residues of newly synthesized proteins translocated across the ER membrane. As folding progresses, two of the glucose residues of the N-linked glycan are trimmed by the glucosidases I and II. The resulting monoglucosylated protein then binds to calnexin and calreticulin. When the remaining glucose residue is trimmed by glucosidase II a protein with the carbohydrate structure $\text{Man}_9\text{GlcNAc}_2$ is released. If the glycoprotein is not properly folded, the N-glycan is reglucosylated at the same position by a UDP-glucose:glycoprotein glucosyltransferase (UGGT) inducing a new cycle of calnexin/calreticulin binding, thus preventing the glycoprotein from escaping the ER. Eventually, if this cycle persists, trimming of a mannose residue by ER α 1,2-mannosidase I will generate a glycan with the structure $\text{Man}_8\text{GlcNAc}_2$ leading to an association of the glycoprotein with the ER degradation-enhancing 1,2 mannosidase-like protein (EDEP) (Hosokawa et al. 2001; Jakob et al. 2001). This interaction extracts the misfolded glycoproteins from the calnexin/calreticulin cycle accelerating ERAD (Molinari et al. 2003; Oda et al. 2003). Since yeast lacks UGGT activity (Parodi 1999) it is suggested that the calnexin/calreticulin cycle is a feature of the ER QC that has been acquired more recently in evolution.

Notwithstanding the well-established connection between ER folding and the activity of the ER chaperones, the mechanisms determining how folding attempts are interrupted to target unfolded and misfolded proteins for ERAD remain largely unclear.

Export of ER clients via the secretory pathway

ER exit of mature proteins occurs only when all the QC standards are met. In mammalian cells, COPII vesicles bud from restricted exit sites, referred to as transitional ER (Palade 1975). Two classes of ER clients can be identified in respect to their incorporation into cargo vesicles. While many ER clients may be randomly incorporated into these vesicles, representing bulk flow from ER to Golgi (Wieland et al. 1987), others require export receptor proteins to mediate their exit from the ER. For instance, the mannose specific lectin ERGIC53 specifically recruits glycoproteins to COPII vesicles (Appenzeller et al. 1999). In addition, signals on the cytosolic

side of membrane spanning proteins can also mediate ER exit. LXXL/ME (where X represents any residue) and YNNSNPF motifs found in SNARE proteins are recognized by the COPII component Sec24, acting as export signatures promoting vesicular budding and trafficking (Mossessova et al. 2003).

Incorporation of properly folded proteins in COPII vesicles constitutes the first step of a controlled intracellular transport chain that ultimately delivers the former ER clients to their final destinations.

Protein misfolding and ERAD – on the road to degradation

Not all the proteins entering the ER successfully bypass the complex QC checkpoints and follow their journey throughout the secretory pathway. Either due to conformation-threatening mutations, nutrient deficiency, oxidative stress, toxic substances or other stressful circumstances, some ER clients misfold beyond rescue and leave this compartment through the back-door: the ERAD pathway (Figure 3).

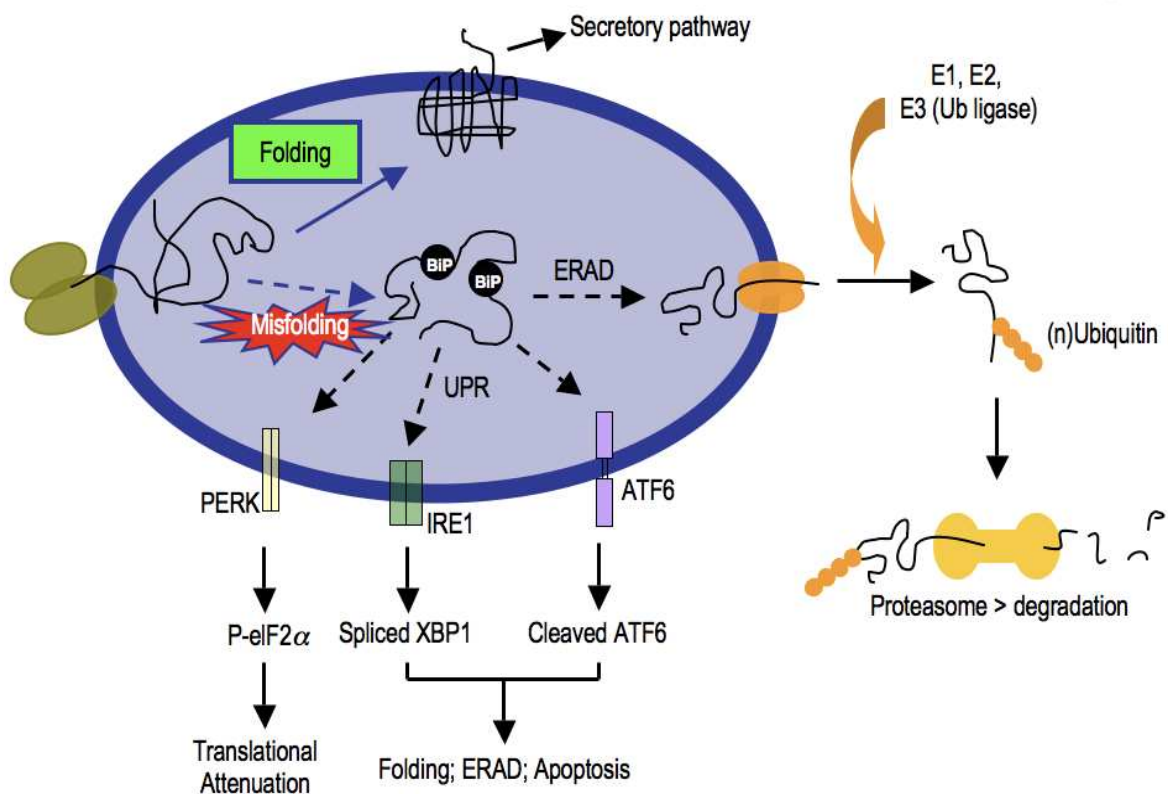


Figure 3 – Fate of the proteins upon entering the ER. Membrane proteins and proteins destined to the secretory pathway enter the ER where, with the assistance of a number of chaperones and enzymes, folding takes place. In addition to the protein pool that successfully acquire a correct conformation and proceed their trafficking beyond the ER, some ER clients fail to bypass the rigorous quality control mechanisms and are targeted to proteasomal degradation. Eventually, if the misfolded products accumulate beyond the ER folding capacity, the unfolded protein response (UPR) is triggered (see text for a detailed explanation).

When proteins are unable to attain their native conformation they are recognized and targeted to degradation. This process, known as ERAD (McCracken and Brodsky 1996; Ellgaard and Helenius 2003),

involves recognition of misfolded proteins, dislocation of these proteins from the ER (Wiertz et al. 1996a; Wiertz et al. 1996b), and their final degradation by the ubiquitin-proteasome system (Tsai et al. 2002; Ellgaard and Helenius 2003; Kostova and Wolf 2003). As elimination of ER proteins that have failed to bypass the ER quality control checkpoint requires the cytosolic ubiquitin-proteasome system, a crucial step in ERAD is the retrotranslocation of the misfolded proteins out of the ER, a process referred to as dislocation (Wiertz et al. 1996a; Wiertz et al. 1996b). As mentioned previously (see Chapter 1 – Part I, “*Hijacking MHC-I: viruses take control*”), the pathways used by HCMV to eliminate MHC-I HCs are among the best studied examples of membrane glycoprotein dislocation (Ahn et al. 1996; Wiertz et al. 1996a). Two small HCMV-encoded membrane glycoproteins, US2 and US11, play a decisive role in this process. During US11 mediated dislocation, Derlin-1 receives the MHC-I HCs from US11, which is involved in substrate recognition in the ER lumen. Derlin-1 also associates with SEL1L, an essential component of the dislocation complex (Mueller et al. 2006) and with the cytosolic ATPase p97, an interaction that is mediated by VCP-interacting membrane protein (Ye et al. 2004). p97 then uses ATP hydrolysis to pull the MHC HCs into the cytosol (Meusser et al. 2005). In the cellular pathway, independently of US11 activity, dislocation may occur through a similar mechanism, but this time Derlin-1 receive the misfolded proteins from ER chaperones (Tsai et al. 2002).

The ubiquitin-proteasome system: a tag to destruction

Once in the cytosol, N-glycanases catalyze the removal of the N-linked glycans from ERAD substrates (Blom et al. 2004; Hirsch et al. 2004). To allow proper recognition by the proteasome, ERAD substrates must acquire a polyubiquitin chain. This process is catalyzed by the combined action of three enzymes: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3) (Meusser et al. 2005). E3s participate in the final step of ubiquitination, transferring the activated ubiquitin from an E2 to a lysine residue of the substrate. While the number of different E1 and E2 is rather limited, E3s are much more abundant. E3s recognize specific ubiquitination signals, which most likely include exposed hydrophobic segments that are normally buried in the native structure of the protein. The final step in ERAD is the delivery of polyubiquitin-marked proteins to the proteasome. The substrate is then degraded to short peptides, followed by a release of free and reusable ubiquitin molecules (Glickman and Ciechanover 2002).

The unfolded protein response

The importance of an efficient folding machinery and ER quality control mechanisms is supported by the existence of an exclusive signal transduction pathway that reacts to the accumulation of unfolded or misfolded proteins in the ER: the unfolded protein response (UPR). Under certain circumstances, for instance due to increased folding demands (e.g. B cell differentiation into Ab secreting plasma cells), genetic mutations, infection by viruses, glucose starvation or hypoxia, the ER folding machinery and/or ERAD components are flooded by faulty proteins (Ma and Hendershot 2004; Wu and Kaufman 2006). To cope with this burst of “unwanted” ER clients, the UPR is activated, enhancing the levels of proteins that participate in folding, ER QC and ERAD and

reducing the rate of protein synthesis. Eventually, if the folding errors in the ER can no longer be overcome, the UPR may induce pro-apoptotic programs (Schroder and Kaufman 2005c) (Figure 4).

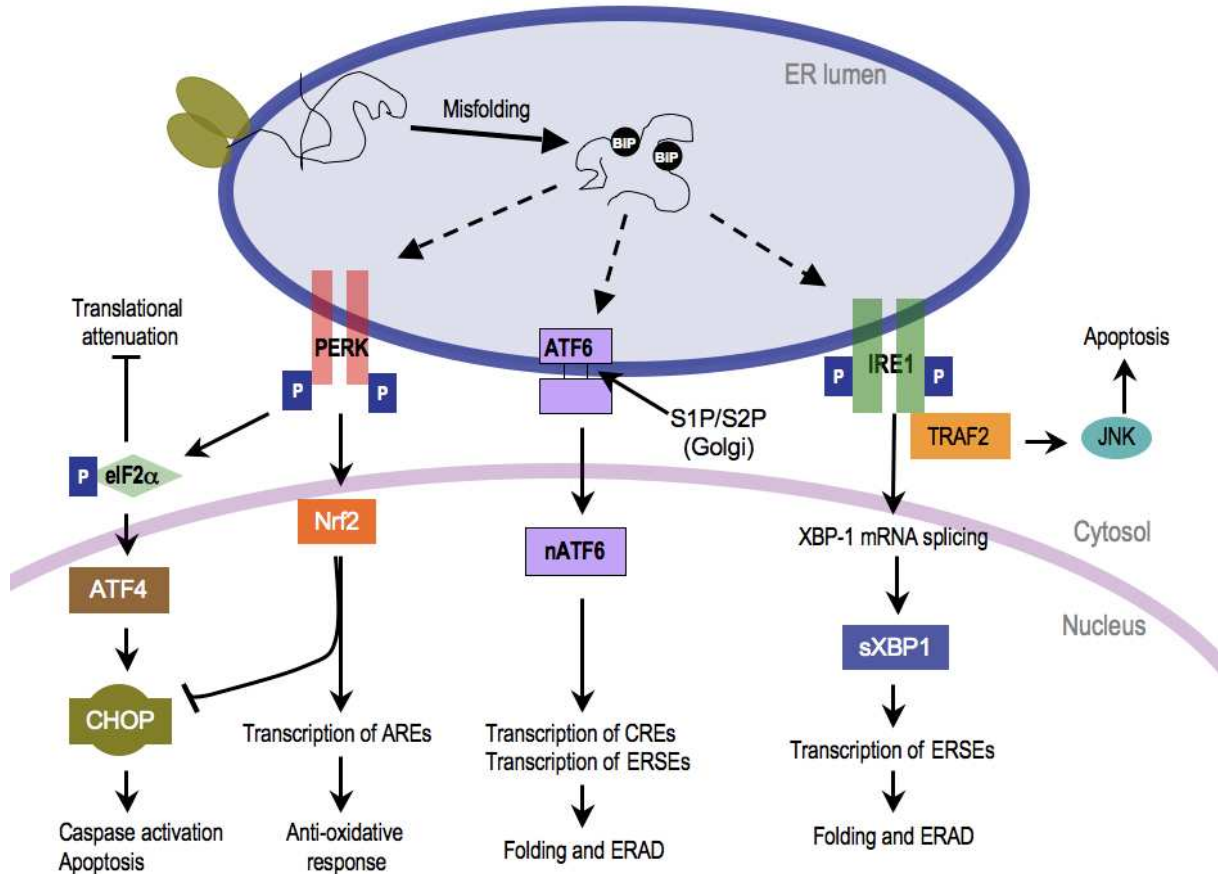


Figure 4 – The unfolded protein response in mammalian cells. Binding of BiP to unfolded proteins in the ER leads to the activation of PERK, ATF6 and IRE1. PERK activation results in eIF2 α -mediated repression of general translation, but by allowing ATF4 translation, it also leads to transcriptional upregulation of some genes, like CHOP. NRF2 phosphorylation by PERK and its translocation to the nucleus promotes the transcription of genes with antioxidant response elements (AREs) in their promoters. Activated ATF6 translocates into the Golgi where, by the action of S1P and S2P proteases, the N-terminal cytoplasmic domain of ATF6 (nATF6) is cleaved. The transcriptionally active nATF6 translocates to the nucleus increasing the transcription of genes with cyclic AMP response elements (CREs) or ER stress response elements (ERSEs). Activation of IRE1 leads to the alternative splicing of the mRNA for the XBP-1 transcription factor. IRE1 also binds TRAF2, activating the pro-apoptotic JNK mitogen activated kinase pathway.

One major sensor and three distinct transducers

BiP is a key UPR sensor. In the absence of stress conditions BiP binds to a number of ER-stress signal transducing proteins: double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring kinase 1 (IRE1). This interaction keeps them inactive until the accumulation of unfolded proteins sequesters BiP and leads to activation of the ER-stress transducers, each

contributing via unique transcription factors to the execution of the transcriptional response (Bertolotti et al. 2000; Zhang and Kaufman 2004).

The PERK pathway

In unstressed cells, BiP associates with the luminal domain of PERK, a type I transmembrane kinase, making it inactive. Upon accumulation of unfolded proteins in the ER, BiP is competitively titrated from PERK resulting in oligomerization and activation of this ER stress signal transducer (Bertolotti et al. 2000). Activated PERK has kinase activity and amongst their substrates are eIF2 α (Harding et al. 1999) and the transcription factor Nrf2 (Cullinan et al. 2003).

One of the hallmarks of the UPR activation - general translation attenuation – results from phosphorylation of eIF2 α by PERK (Harding et al. 1999). Other events following phosphorylation of eIF2 α include loss of cyclin D1, which leads to cell cycle arrest in G1 (Brewer et al. 1999), and the selective translation of ATF4, which contributes to the expression of genes involved in amino-acid import, glutathione biosynthesis, and resistance to oxidative stress (Scheuner et al. 2001; Harding et al. 2003).

Phosphorylation by PERK results in Nrf2 nuclear localization and activation of transcription through the antioxidant response element (ARE) (Venugopal and Jaiswal 1998; Nguyen et al. 2000). The ARE controls expression of several genes such as the glutathione S-transferase, NAD(P)H:quinone oxidoreductase, γ -glutamylcysteine synthetase, and UDP-glucuronosyl transferase. Nrf2 is associated with the cell's ability to mount an anti-oxidative response, preventing the accumulation of reactive oxygen species in cells exposed to ER stress (Cullinan et al. 2003).

The ATF6 pathway

A second branch of the UPR is mediated by ATF6. ATF6 is a bZIP transcription factor, but does not exert its task until the UPR is triggered. As for PERK, ATF6 regulation involves the binding/release of BiP (Shen et al. 2002). However, rather than regulating the activity of oligomerization domains, BiP manages the activity of two independent and redundant Golgi localization sequences, GLS1 and GLS2. In unstressed cells, BiP retains ATF6 in the ER by inhibiting its GLSs and dissociation of BiP during ER stress allows ATF6 to be transported to the Golgi, where activation takes place (Shen et al. 2002). Additionally, ATF6 retention in the ER involves its interaction with calreticulin (Hong et al. 2004). Upon ER stress, newly synthesized ATF6 is underglycosylated, which abrogates its interaction with calreticulin and allows it to migrate to the Golgi apparatus. Here, by the combined action of two proteases (S1P and S2P), a nuclear-targeted fragment (nATF6) bearing the transcription factor activity is severed proteolytically from the membrane bound ATF6 (Ye et al. 2000). nATF6 activates transcription from promoters containing cyclic AMP responsive element (CRE) (Wang et al. 2000) or ER stress response elements (ERSE-I and ERSE-II) (Yoshida et al. 1998; Li et al. 2000; Kokame et al. 2001; Okada et al. 2002). Some of the transcriptional targets of ATF6 are BiP, GRP94, X box-binding protein-1 (XBP-1) and C/EBP homologous protein (CHOP) (Yoshida et al. 2000).

The involvement, even if indirectly, of the calnexin/calreticulin cycle and BiP recognition of unfolded proteins on the activation of the ER stress transducer ATF6, establishes a cross-talk between the ER QC machinery and the UPR activation. This mechanistic interplay may provide a more efficient response to the incidence of ER stress conditions.

The IRE1 pathway

IRE1 is a type I transmembrane protein with kinase and endoribonuclease (RNase) domains (Cox et al. 1993; Mori et al. 1993). Its activation proceeds through a mechanism resembling PERK activation. In the inactive state, the luminal domain of IRE1 associates with BiP, which is released upon accumulation of unfolded proteins in the ER lumen (Bertolotti et al. 2000). Driven by its ER-luminal dimerization domain, BiP-free IRE1 homodimerizes, autophosphorylates and activates its RNase domain (Bertolotti et al. 2000; Liu et al. 2003). The substrate for the RNase is XBP-1. Activation of the UPR causes IRE1-dependent splicing of a small intron from the XBP-1 mRNA (Calfon et al. 2002). The splicing reaction introduces an alternative C terminus with increased transcriptional activation potential in XBP-1. Transcriptionally active XBP-1 controls the expression of genes that are mediated by ERSE, like BiP (Yoshida et al. 2001).

In an example of cooperation between distinct UPR branches, both IRE1 and ATF6 signalling pathways merge through regulation of XBP-1 activity. Whereas nATF6 increases the amount of XBP-1 mRNA, IRE1 drives the splicing reaction that increases XBP-1 transactivation potential (Lee et al. 2002).

A life-threatening decision: green light to apoptosis

If ER stress continues such that homeostasis is not recovered, cells may commit to apoptosis. Apoptotic programs are turned on by signals from each of the three UPR branches along with Ca^{2+} release from the ER (Scorrano et al. 2003; Zong et al. 2003). For instance, the PERK and ATF6 branches of the UPR both contribute to upregulation of pro-apoptotic genes, such as CHOP, which is under transcriptional control by ATF4 (a target for phosphorylated eIF2 α) (Harding et al. 2000) and nATF6 (Yoshida et al. 2000). By downregulating the expression of Bcl-2 (McCullough et al. 2001; Ma et al. 2002), CHOP promotes mitochondrial cytochrome c release and activation of caspases that lead to cell death. IRE1 activation and binding to TRAF2 is thought to contribute to activation of caspase-12 and caspase-4 by a mechanism involving the c-Jun N-terminal kinase (JNK) cascade (Nakagawa et al. 2000; Urano et al. 2000; Hitomi et al. 2004). Although the link between the accumulation of unfolded proteins and Ca^{2+} release is not clarified, calpain, which responds to Ca^{2+} release from the ER, is thought to participate in the proteolytic activation of caspases (Yoneda et al. 2001). If persistent, the UPR proceeds towards a life/death decision. A delicate balance between cytoprotective and cytotoxic pathways determines the cell's fate: either commit to apoptosis or survive carrying unfolded/malfunctioning proteins. The molecular details behind this choice are not yet fully understood.

OASIS and BBF2H7: introducing the cell type-specific UPR transducers

Recent studies have suggested the existence of other ER stress signal transducers besides the “classical” PERK, ATF6 and IRE1. Focus has been on the investigation of dedicated UPR pathways that fulfil the exclusive demands of each cell type’s response to the ER stress conditions.

A novel ER stress sensor, OASIS, specifically expressed in astrocytes, was recently identified. Like ATF6, OASIS is a transmembrane protein containing a bZIP domain. In response to ER stress, OASIS is cleaved and its amino-terminal cytoplasmic domain translocates into the nucleus where it induces the transcription of target genes that are mediated by CRE and ERSE, thus modulating the unfolded protein response in astrocytes (Kondo et al. 2005).

BBF2 human homolog on chromosome 7 (BBF2H7) is an OASIS homologue, containing a bZIP domain in the cytoplasmic portion, which is cleaved at the ER membrane following ER stress. Cleaved BBF2H7 then travels into the nucleus where it activates transcription of target genes containing a CRE. BBF2H7 protein is mainly induced in neurons where it is thought to play important roles in protecting these cells from the accumulation of unfolded proteins (Kondo et al. 2007).

These recent findings lead to the possibility that cell type-specific UPR signalling transducers homologous to OASIS and BBF2H7 may also be involved in other cell type-specific ER stress responses (Saito et al. 2007).

The Unfolded Protein Response in Health and Disease

The UPR is not an exclusive feature of a pathological condition (Wu and Kaufman 2006). In fact, there is nothing such as a totally unstressed cell and a minimum physiologic level of ER stress is always observed. The finding that cells unable to mount a proper UPR and ERAD are not viable (Travers et al. 2000), consolidate the perception of the UPR as a survival mechanism. On the other hand, this specialized ER stress response is often associated with the progression of several pathological conditions, such as genetic diseases, cancer or diabetes (Schroder and Kaufman 2005b).

The UPR in physiological processes

Several specific roles of the UPR on diverse developmental and metabolic processes have been described so far (Wu and Kaufman 2006). Cellular processes, such as regulation of differentiation require the UPR to tightly regulate the biosynthetic burden and the folding capacity of the ER (Schroder and Kaufman 2005a).

More specialized events also imply the participation of the UPR as observed when a high level of protein synthesis is required in secretory cells. This forces the need for a competent protein folding and secretion mechanism to be available for which activation of specific pathways of the UPR is vital. Plasma cells, pancreatic β -cells, hepatocytes, and osteoblasts are among the professional secretory cells whose function is strictly dependent on a functional UPR (Wu and Kaufman 2006). The differentiation of B-Lymphocytes into antibody-secreting plasma cells constitutes a particularly well-studied example of the involvement of the UPR in a crucial

physiological process (Gass et al. 2002; Iwakoshi et al. 2003). A similar need for a specific pathway of the UPR is present in glucose-regulated synthesis of proinsulin in pancreatic β -cells, where fluctuations of glucose levels shut the PERK-mediated inhibition of proinsulin mRNA translation on or off (Scheuner et al. 2001). In low glucose conditions the UPR is activated since impaired protein glycosilation and ATP production disturb protein folding in the ER. This inhibits translation of proinsulin mRNA. Increasing glucose levels eventually restore the folding capacity of the ER and the UPR is inactivated, shutting off the PERK-mediated inhibition of proinsulin mRNA. Accordingly, mice with a deletion mutation of p58IPK, a negative regulator of PERK, have been found to exhibit a phenotype reminiscent of type 1 diabetes (Ladiges et al. 2005). These observations illustrate the dependency of the cellular homeostasis on a functional and fully capable UPR.

Table 1 – Human diseases directly linked to ER stress

Disease	Protein	Function
Wolcott-Rallison syndrome	PERK	UPR sensor
Wolfram syndrome	WFS1	ERAD
Hereditary tyrosinemia type 1	FAH	Tyrosine metabolism
Familial hypercholesterolemia	LDLR	Low density lipoprotein receptor
Z α 1-antitrypsin deficiency	α 1-antitrypsin	Protease inhibitor
Inclusion body myopathy	P97/VCP/CDC48	ERAD
Parkinson's disease	Parkin	E3 ubiquitin ligase
Familial Alzheimer's disease	Presenilin-1	g-secretase complex
Familial Amyotrophic Lateral Sclerosis	SOD1	Cu/Zn superoxide dismutase
Marinesco-Sjogren syndrome	SIL1/BAP/SLS1	BiP adenine nucleotide exchange factor
GM1 gangliosidosis	β -Galactosidase	Carbohydrate/lipid metabolism
Pelizaeus-Merzbacher disease	PLP1	Proteolipid protein 1, myelin component
Batten disease/Infantile neuronal ceroid lipofuscinosis	PPT1	Palmitoylprotein thioesterase-1
Bipolar disease	XBP-1	UPR sensor
Diabetes (Akita mouse)	Proinsulin-2	Insulin precursor
Transmissible Spongiform Encephalopathy	PrP	
Spinocerebellar ataxia 3/Machado-Joseph disease	SCA3	
Huntington's disease	Huntingtin	
Sporadic inclusion body myositis	APP	

Modified from: (Zhao and Ackerman 2006).

The involvement of the UPR in pathological conditions: friend or foe?

Although the primary function of the UPR is to protect cells from insults on the cellular protein folding and secretory capacity, it is increasingly patent that a number of pathological conditions can also activate the ER stress response (Table 1), which, at least in some cases, seems to contribute to the disease processes. However, it remains unclear whether activation of the UPR observed in a range of different disease models favours the organism protection or the disease progression.

Diabetes

The Akita mouse provides one particularly well-studied example of a pathologic effect of the UPR. This mouse has a Cys96Tyr mutation in the proinsulin-2 gene and develops a diabetic phenotype (Yoshioka et al. 1997; Wang et al. 1999). The resulting mutant protein does not fold correctly and is retained in the ER of pancreatic β cells leading to the UPR activation. The continuous misfolded protein accumulation provokes the initiation of apoptotic programs linked to the UPR. If bred into a CHOP knockout background, decreased pancreatic β cell death and delayed onset of diabetes are observed in the Akita mouse (Oyadomari et al. 2002), suggesting that UPR activation and β cell death, rather than the lack of insulin secretion, are the main contributors to the disease progression. Moreover, a recent study revealed that ER stress might also be a key player in obesity-induced type 2 diabetes (Ozcan et al. 2004).

Tumour development

Activation of the UPR has been coupled with the restricted supply of nutrients and oxygen present in tumours, which impinge on the normal maturation of secretory proteins. However, whether the UPR contributes to cancer cell survival and tumour development or if it is associated with cell death through activation of apoptotic components is not clear (Ma and Hendershot 2004).

Neurodegenerative diseases

Upregulation of ER stress markers has been observed in several neurodegenerative diseases, including Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis and polyglutamine (poly(Q)) expansion diseases such as Huntington disease and spinocerebellar ataxias (Zhao and Ackerman 2006). UPR activation in these diseases is often associated with mutations leading to protein misfolding, the formation of irregular protein inclusions or fibrillar aggregates (Kakizuka 1998; Oyadomari and Mori 2004). Despite the unclear significance of the UPR on the progression of these diseases, previous studies suggest that ER stress may be tightly correlated with neuron death through activation of apoptotic programs (Zhao and Ackerman 2006). ER stress and JNK-mediated caspase-12 activation have been previously associated with poly(Q) aggregates, suggesting that stimulation of the UPR may be involved in the pathogenesis of neurodegenerative disorders involving poly(Q) expansion (Kouroku et al. 2002).

CHAPTER 2

HFE CROSS-TALKS WITH THE MHC CLASS I ANTIGEN PRESENTATION PATHWAY

de Almeida SF, Carvalho IF, Cardoso CS, Cordeiro JV, Azevedo JE, Neefjes J, de Sousa M. *Blood*.
2005 Aug 1;106(3):971-7.

HFE is a protein known to be involved in iron metabolism yet, besides its homology with major histocompatibility complex (MHC) class I molecules, it has not been described as having an immunological function. Here we report that peripheral blood mononuclear cells (PBMC) from Hereditary Hemochromatosis (HH) patients carrying the C282Y mutation in HFE have reduced cell surface expression of MHC class I due to enhanced endocytosis rate of MHC class I molecules caused by premature peptide and β_2m dissociation. This faster turnover also leads to increased expression levels of cell surface free class I HC in mutant PBMC. Biochemical analysis indicates an earlier peptide loading and endoplasmic reticulum maturation of MHC class I molecules in C282Y mutant cells. Thermostability assays further showed that in HFE mutants the MHC class I peptide loading gives rise to low stability heterotrimers that dissociate prematurely during its intracellular traffic. The present results suggest the existence of an intriguing cross-talk between a particular HFE mutation and the classical MHC class I route. These findings constitute the first description of peptide presentation pathway abnormalities linked to HFE and provide additional evidence for the occurrence of immunological defects in HH patients.

Antigenic peptides presented by major histocompatibility complex (MHC) class I molecules to CD8⁺ T cells are mainly derived from proteolysis of newly synthesized proteins in the cytosol (Rock and Goldberg 1999; Reits et al. 2000; Schubert et al. 2000) by the proteasome and TPPII (Reits et al. 2004). A heterodimer of two membrane spanning molecules, termed TAP1 and TAP2 translocates the peptides to the lumen of the endoplasmic reticulum (ER) (Androlewicz et al. 1993; Neefjes et al. 1993a; Shepherd et al. 1993; Androlewicz and Cresswell 1994) where they undergo further trimming of their amino-terminal ends by the aminopeptidase ERAAP (Saric et al. 2002; Serwold et al. 2002). Loading of peptides onto MHC class I molecules involves the formation of a transient protein complex termed the peptide loading complex (PLC) (Cresswell et al. 1999; Cresswell 2000) that facilitates the binding of peptide to MHC- β_2m -microglobulin (β_2m) heterodimers (Cresswell et al. 1999). The conformational changes that occur in the newly formed peptide loaded MHC class I molecules precede their release from the PLC and subsequent delivery to the cell surface via the standard secretory pathway.

MHC class I assembly and export from the ER is a complex process (Marguet et al. 1999; Spiliotis et al. 2000) subjected to a set of quality control mechanisms. This orchestrated process acts concertedly to ensure that high affinity peptides are loaded onto class I molecules. Tapasin, calreticulin and ERp57 are some of the key players supposed to take part in this event (Paulsson and Wang 2004). Yet the precise mechanisms responsible for optimization of class I maturation and for the prevention of ER escape of premature class I molecules remain to be fully understood.

Here, we have studied assembly, peptide loading and surface expression of MHC class I molecules in peripheral blood mononuclear cells (PBMC) of either control blood donors or Hereditary Hemochromatosis (HH) patients. HH is a disease characterized by the excessive deposition of iron in several organs and is the most common autosomal recessive disorder in the Caucasian population (Merryweather-Clarke et al. 1997). The gene for HH, HFE, is a 343 residue type I transmembrane glycoprotein homologous to MHC class I proteins that also interacts with β_2m (Feder et al. 1996). However, despite the structural similarities with MHC class I molecules, HFE lacks a functional peptide binding groove (Lebron et al. 1998). An HFE mutation, in which Cysteine at position 282 was changed to Tyrosine (C282Y), has been identified in the majority of HH patients (Feder et al. 1996). The C282Y mutation prevents the formation of an intra-molecular disulfide bond in the $\alpha 3$ domain of HFE and abrogates β_2m association and cell surface expression of the protein influencing its effects on the regulation of cellular iron levels (Feder et al. 1997; Waheed et al. 1997; Roy et al. 2000). The observation that HFE binds to Transferrin Receptor I (Parkkila et al. 1997a; Feder et al. 1998), an homodimeric cell surface glycoprotein which acts as the receptor for iron-loaded Transferrin (Leibman and Aisen 1977), implicated HFE in the regulation of iron homeostasis.

The previous observations that HH patients have low numbers of CD8⁺ T cells (Reimao et al. 1991; Porto et al. 1997), decreased CD8-p56lck activity (Arosa et al. 1994), the anomalies in the T-cell receptor repertoire observed in C282Y mutation carriers (Cardoso et al. 2001) and the diminished cytotoxic activity of HH patients' CD8⁺ cytotoxic T lymphocytes (CTL) (Arosa et al. 1997) established a solid link between the immunological system and the iron metabolism. Yet, the key player, if there is one, involved in this intriguing

association remains to be unrevealed. Here we show that MHC class I surface expression is decreased in HH patients PBMC with the HFE C282Y mutation. Metabolic labeling experiments showed that the proportion of properly assembled class I molecules and unfolded MHC class I heavy chains (HC) are significantly different between control and HFE C282Y mutated PBMC. In view of these findings we analyzed the peptide loading kinetics, stability and the maturation of MHC class I molecules. We describe an association between a mutation in the HFE gene and anomalies in the assembly, maturation and surface expression of MHC class I molecules.

Material and Methods

Antibodies The following Abs were used: The mAb W6/32 (Dako, Glostrup, Denmark); HC-10; Anti-HLA-A,B,C-FITC conjugated mAb clone G46-2.6 (BD Pharmingen, San Diego, CA); Anti-MHC class II alpha-chain; 8C-10 (Mouse anti-human HFE) that recognizes HFE/ β_2m heterodimers (a kind gift from Dr Rachel Ehrlich, Department of Cell Research and Immunology, Tel Aviv University, Israel) and Rabbit anti-HFE cytoplasmic tail (CT) that recognizes free HFE HC (Ben-Arieh et al. 2001); Donkey-anti-Mouse-FITC conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA); Anti-CD14-RPE clone TUK4, Anti-CD8-FITC clone DK25 and anti-CD4-PE clone MT310 (Dako, Glostrup, Denmark);

Cells PBMC were obtained from healthy blood donors and Hereditary Hemochromatosis patients at the HH Clinic, HGSA, Oporto, after informed consent, according to the Declaration of Helsinki. When possible, in each individual experiment subjects were matched by sex and age. After centrifugation over Lymphoprep (Nycomed, Oslo, Norway), PBMC were washed with HBSS. Contaminating RBCs were lysed in 10 mM Tris, 150 mM NH_4Cl , pH 7.4 at 37°C for 10 minutes.

HFE genotyping HFE genotyping was done using the Haemochromatosis StripAssayB kit (ViennaLab, Vienna, Austria) according to the manufacturer's protocol, as described previously (Cardoso et al. 2001).

Flow cytometry For HFE intracellular staining 1×10^6 PBMC were fixed by incubation in PBS containing 4% formaldehyde for 15 minutes at room temperature. Fixed cells were incubated for 30 minutes on ice with 8C10 (diluted in PBS, 0.2% BSA, 0.1% NaN_3 , 0.2% Saponin), washed twice with PBS and incubated with Anti-mouse-FITC for 30 minutes. Cells were washed 3 times and immediately acquired in a FACScalibur (Becton Dickinson, Mountain View, CA). For surface immunofluorescence staining, 1×10^6 PBMC were washed in ice cold PBS, 0.2% BSA, 0.1% NaN_3 followed by incubation at 4°C with a saturating amount of primary Ab for 30 minutes in 96-well plates. After 3 washes cells were incubated with secondary Ab for 30 minutes on ice. Cells were washed twice and FACS analysis performed in a FACScalibur. For the class I stability assay, cells were either incubated overnight at 25°C and 37°C in RPMI with Glutamax, 10% FBS (figure 7B) or cultured for 5 hours in the presence of 20 $\mu g/ml$ Brefeldin A (BFA) (Affiniti, Exeter, UK) (figure 7C) before surface staining with W6/32. For each sample a minimum of 10 000 events were acquired. To define the background staining irrelevant mAbs of the same isotype were used.

Metabolic Labeling and Endo H digestion 5×10^6 PBMC were starved for 1 hour in Cysteine/Methionine free DMEM medium supplemented with 1% L-glutamine and labeled for 2.5 hours with 140 $\mu Ci/ml$ Pro-Mix L- $[^{35}S]$ Cysteine/Methionine (Amersham Biosciences UK, England). After labeling, cells were washed three times with HBSS and lysed in ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Triton-X, Complete EDTA Free-protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 10 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO)). The nuclei and cell debris were removed by centrifugation and the lysates pre-cleared with 100 μl protein-A Sepharose beads slurry (50%) for 1 hour at 4°C. Half of the lysates corresponding

to 2.5×10^6 cells containing equivalent amounts of TCA-precipitable radioactivity was incubated at 4°C with HC-10 and the other half with W6/32 under the same conditions. Immunocomplexes were pulled down with protein-A sepharose beads and washed three times in ice-cold lysis buffer. For HLA-DR immunoprecipitation 5×10^6 cells were labeled and processed as described. In the thermostability experiments pre-cleared cell lysates were incubated at 0°, 25°, 37° and 42°C for 3 hours before immunoprecipitation with W6/32. For Endo H assay, the W6/32 immunocomplexes were divided into 3 equal portions: one was left untreated (negative control), the others were digested for 6 hours at 37°C with Endo H (Roche Diagnostics, Mannheim, Germany) or n-Glycosidase F (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer. The reaction was stopped by addition of gel loading buffer solution (Laemmli 1970) with 10% β -mercaptoethanol and boiling for 5 minutes.

Pulse chase experiments to follow peptide loading Experiments were performed as described previously (Neefjes et al. 1993b). Briefly 50×10^6 PBMC were starved in Cysteine/Methionine free medium and labeled for 5 minutes with 140 μ Ci/ml L-[³⁵S]Cysteine/Methionine. The labeling was stopped by the addition of 1 mM cold Methionine and Cysteine (Sigma-Aldrich, St. Louis, MO) and the cells were chased for 0, 1, 2, 4 and 6 minutes at 26°C. At every chase time 200 μ l of cell suspension were injected into 2 ml of ice-cold lysis buffer. After one hour on ice the cell debris was removed by centrifugation and the lysates pre-cleared as described above. Cell lysates were split into two equal portions. One was left on ice and the other was incubated at 37°C for 1 hour. MHC class I molecules were then immunoprecipitated from lysates with equivalent amounts of TCA-precipitable radioactivity with W6/32.

SDS-PAGE and Quantitation 10% SDS-PAGE was performed using a Bio-Rad Mini Protean II kit. Gels were fixed in 10% acetic acid, 40% methanol, incubated for 30 minutes with Amplify solution (Amersham Biosciences UK, England) dried and exposed to a radioactivity storage screen. Quantitation was performed using a Typhoon phosphoimager (Molecular Dynamics, Sunnyvale, CA) with ImageQuant v5.1 software.

Western Blot Total protein concentration from whole cell lysates was determined with RC/DC Protein Assay (Bio-Rad, Hercules, CA) and 30 μ g separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose Hybond-C membrane (Amersham Biosciences UK, England). After blocking at 4°C with 5% dry milk/0.05% Tween 20 in PBS (PBS-T), the membrane was incubated with anti-HFE (CT), washed 3X with PBS-T and detected with Goat anti-Rabbit-IgG-HRP conjugate (Molecular Probes, Eugene, OR) and the enhanced chemiluminescence substrate Super Signal West Dura (Pierce, Rockford, IL).

Statistical analysis To test the significance of the differences observed in figures 5, 6 and 7, the Student's T-test was used. In all tests the statistical significance was 2-sided and considered at $p < 0.05$. Analysis of flow cytometry results of cells surface MHC class I (figure 2) was done plotting the Gaussian Cumulative Distribution Function (CDF) of the Mean Fluorescence Intensity (MFI) observed in each individual experiment using SigmaPlot v8.02.

Statistical differences were calculated with the Kolmogorov-Smirnov test. In figures 5, 6 and 7 data are displayed as mean $\pm$ 1 SD.

Results

Reduced cell surface expression of β_2m assembled MHC class I molecules and increased free MHC class I HC in C282Y mutants

The tissue expression pattern of HFE is still subject to intensive study. In that context we decided to check whether this protein is present in PBMC by flow cytometry and also by western blot analysis. Using an anti-HFE antibody that labels the properly folded HFE molecules, and thus does not detect C282Y mutant forms, HFE wild type (WT) cells were permeabilized, labeled and analyzed by fluorescence-activated cell sorting (FACS) (Figure 1A). For western blot analysis whole cell lysates from WT PBMC were processed as described using an antibody that recognizes free HFE HC (HFE CT) (Figure 1B). The results of both experiments revealed that HFE is expressed in PBMC.

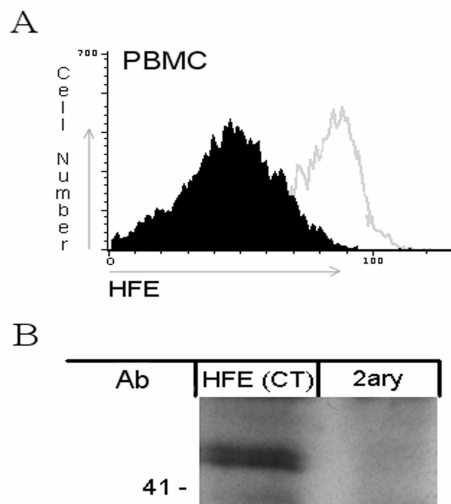
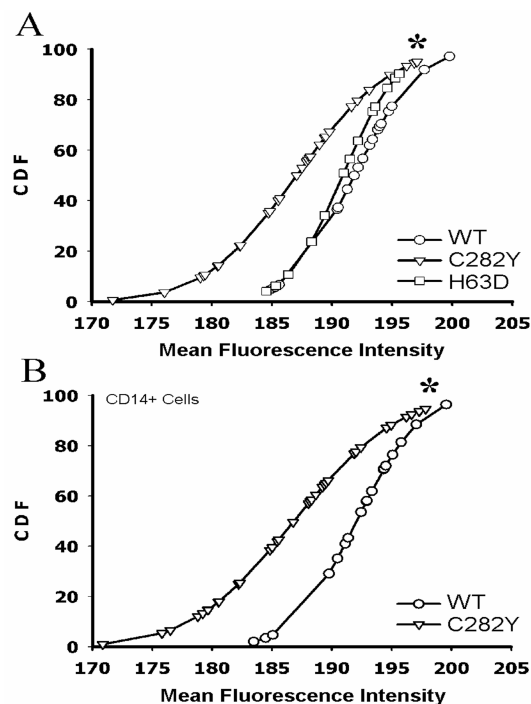


Figure 1 - HFE expression in PBMC. (A) Permeabilized PBMC (without the C282Y HFE mutation) were stained for HFE. The open area represents the HFE labeling in WT cells. The filled area represents the mean fluorescence intensity (MFI) negative control obtained by incubation with an isotype-matched non-specific antibody. A representative example of seven independent experiments is shown. (B) HFE from whole cell lysates of WT PBMC was detected by Western blot with anti-HFE C-terminal (CT) Ab. 30 μ g of total protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti-HFE (CT) or with secondary antibody alone (2ary). The molecular weight marker is shown on the left.

To assess the effect of the C282Y mutation on the cell surface expression of MHC class I we used PBMC isolated from C282Y mutants HH patients and from control blood donors without that particular mutation (WT). FACS analysis revealed significantly lower cell surface labeling with the anti-HLA-A,B,C-FITC mAb (recognizes assembled MHC class I) in C282Y mutated PBMC than in WT cells (Figure 2A). We have also analyzed PBMC heterozygous for the H63D mutation in HFE, a mutation also seen in some HH patients (Feder et al. 1996). In this case staining with anti-HLA-A,B,C-FITC did not show any significant difference in the MHC class I surface expression when compared to WT cells ($p>0.8$) (Figure 2A). Since PBMC are a rather heterogenic population, we also analyzed the surface expression of MHC class I molecules in CD14 positive cells (monocytes) only. PBMC were double stained with anti-HLA-A,B,C-FITC and anti-CD14-RPE conjugated mAbs. FACS analysis showed that C282Y monocytes have a reduced surface expression of class I molecules compared to WT monocytes (Figure 2B).

Figure 2 - Reduced cell surface expression of MHC class I molecules in HFE C282Y mutant PBMC. (A) HFE Wild-type (circles), C282Y mutant (triangles) and H63D mutant (squares) PBMC were surface stained for MHC class I. Results are presented as the Gaussian cumulative distribution function (CDF) of the Mean Fluorescence Intensity obtained in each individual experiment. The asterisk represents a statistically significant difference between the MFI distribution of the C282Y mutants when compared to the WT and H63D cells ($D_n=0.53$; $p<0.01$). **(B)** PBMC were double stained with anti-HLA-A,B,C-FITC and anti-CD14-PE mAb. Class I staining MFI in double positive cells is presented as the CDF. C282Y mutant and WT cells are compared. The asterisk represents a statistically significant difference between the MFI distribution of the C282Y mutant cells when compared to the WT cells ($D_n=0.58$; $p<0.001$). Sixty-four individuals were studied with the following distribution: n (WT) = 19; n (C282Y carriers) = 32; n (H63D carriers) = 13.



To evaluate if the observed decrease of β_2m assembled class I molecules in C282Y cells was accompanied by significant changes in the amount of surface expressed free MHC class I HC, non-permeabilized PBMC obtained from C282Y homozygous HH patients and from WT control blood donors were stained with HC-10 (which recognizes only free class I HC) and analyzed by FACS. In contrast to the result obtained for the β_2m assembled class I molecules (Figure 2), the levels of surface expressed free MHC class I HC were higher in C282Y homozygous than in WT PBMC (Figure 3). Again, the comparison of specific cell populations revealed a similar result (Figure 3A vs 3B). To examine the MHC class I specificity of the previous results, CD4 (Figure 3C) and CD8 (Figure 3D) surface staining was performed. No differences were observed in the expression of these two molecules.

All the HH patients were genotyped for MHC class I alleles. No significant correlation was observed between the results obtained and the allelic frequency (see supplementary data – Table S2).

Assembly of MHC class I molecules is altered in C282Y mutants

To examine if the reduced class I cell surface expression in C282Y mutants is due to a decreased efficiency of MHC class I assembly, we analyzed the ratio between MHC class I HC and the β_2m assembled class I molecules isolated from whole cell lysates. ^{35}S -met/cys labeled PBMC with (C282Y) or without (Control) the C282Y mutation were lysed and precipitated using the mAbs HC-10 and W6/32, which showed no cross-reactivity against HFE molecules (data not shown). W6/32 recognizes a conformational epitope located on the $\alpha 1\alpha 2$ domain of the HC and requires correct assembly with β_2m . The result of these experiments (Figure 4A) revealed marked differences in the relative proportions of the β_2m bound and free MHC class I HC. Fewer class I HC and

more β_2m assembled class I molecules were found in C282Y mutant PBMC than in controls, exactly the opposite of that observed at the cell surface (Figures 2 and 3).

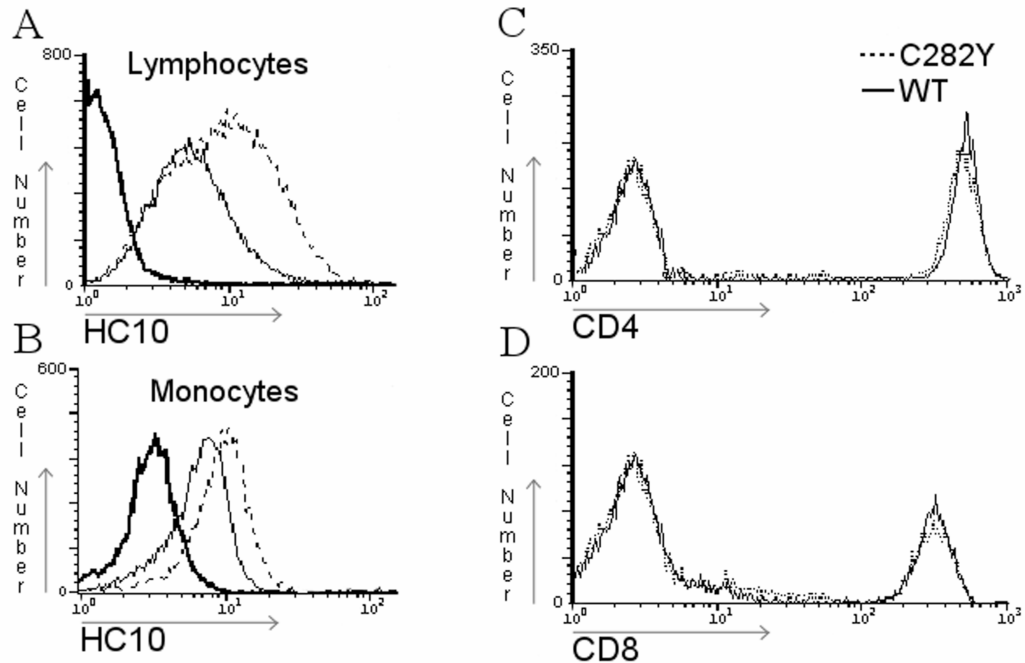


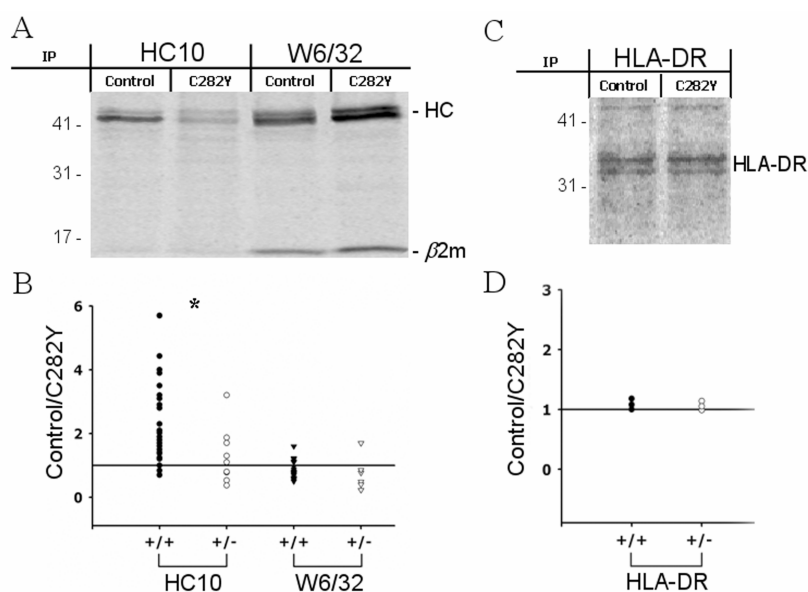
Figure 3 – C282Y mutant cells display higher levels of surface expressed free MHC class I HC. HFE Wild-type or C282Y homozygous PBMC were surface stained with HC10 and analyzed by flow cytometry. Total lymphocytes (A) and monocytes (B) were gated and analyzed separately. Anti-CD4 (C) and anti-CD8 (D) surface staining of WT and C282Y homozygous PBMC was carried out in order to demonstrate the specificity of the result. The dotted line in the four histograms represents the C282Y homozygous staining, the narrow solid line the WT staining and the broad solid line is the negative control obtained by incubation with an isotype-matched non-specific antibody. These are representative data from one experiment repeated five times with similar results.

The ^{35}S labeling experiments were also performed in the presence of the iron chelator deferoxamine (DFO) (supplementary data – Figure S1). Immunoprecipitation with both W6/32 and HC-10 antibodies showed that iron loading is not the cause of the different amounts of assembled and free MHC class I molecules.

Quantitative analysis of the results indicated an HFE allele dependent effect. The Control/C282Y ratio calculated for the intensity of the HC-10 immunoprecipitates was significantly higher in C282Y homozygous than in C282Y heterozygous cells (Figure 4B). In control cells there are, in average, 2.5 times more free MHC class I HC than in C282Y homozygous cells and 1.3 times more than in C282Y heterozygous cells. W6/32 immunoprecipitates did not reveal the same HFE allele dependency (Figure 4B). In this case, the average of the Control/C282Y ratios was 0.77 (C282Y +/+) and 0.74 (C282Y +/-) and no statistical significance was observed.

To prove the specificity of the previous observations, MHC class II alpha chain (HLA-DR) was recovered from whole cell lysates by immunoprecipitation. As depicted in Figures 4C and 4D, no differences were found in the amount of immunoprecipitated protein between the control and the C282Y cells.

Figure 4 – β_2m assembled and free MHC class I HC levels are altered in C282Y mutant cells in an allele dependent manner. (A) PBMC with (C282Y) or without (Control) the C282Y mutation were ^{35}S labeled for 2.5 hours and class I molecules recovered with HC-10 and W6/32 mAbs and subjected to 10% SDS-PAGE. The position of the MHC class I HC and β_2m are indicated. Molecular weight markers are shown on the left. Data shown represent one individual experiment using PBMC from one WT blood donor and one C282Y homozygous HH patient of a total of 40 studied. (B) MHC class I HC bands were quantified by phosphoimaging. In each individual experiment one control blood donor and one HH patient carrying the C282Y mutation were tested. The intensity of the control bands was divided by the C282Y bands. The ratios were plotted according to the patient's HFE genotype: C282Y homozygous (+/+) - closed circles/triangles; C282Y heterozygous (+/-) - open circles/triangles. (*) $p < 0.05$. The means $\pm$ SD of 40 experiments with the following distribution are shown: HC-10 (C282Y +/+) = 31; HC-10 (C282Y +/-) = 9; W6/32 (C282Y +/+) = 23; W6/32 (C282Y +/-) = 6. (C) As a control experiment, HLA-DR was recovered from control and C282Y ^{35}S labeled PBMC and resolved on a 10% SDS-PAGE. (D) The Control/C282Y ratios of the bands intensity were plotted according to patient's HFE genotype. Data are representative of 6 individual experiments.



C282Y carriers load MHC class I more efficiently than WT subjects

To investigate the peptide loading kinetics of MHC class I in C282Y homozygous compared to control PBMC, cells were pulse labeled for 5 min and chased for the indicated times (Figure 5). After lysis three different conformations of class I molecules were considered; class I HC recognized by the mAb HC-10, class I HC/ β_2m heterodimers and class I HC/ β_2m /peptide heterotrimers both recognized by the mAb W6/32. Peptide binding confers thermal stability to class I heterodimers (Schumacher et al. 1990; Townsend et al. 1990; Baas et al. 1992) avoiding their dissociation. Therefore, after incubation at 37°C only class I heterotrimers were isolated by W6/32. By comparing the amount of protein recovered after the incubation at 37°C with that recovered from the lysates maintained on ice, the assembly of class I heterotrimers *in vivo* can be analyzed. The total amount of class I heterodimers and heterotrimers recovered with W6/32 at 0°C increased at every time point, both for control and C282Y cells (Figure 5A, W6/32-0°C). The similar amount of immunocomplexes recovered at 0°C demonstrates that class I HC/ β_2m heterodimers formation occurs with analogous kinetics and excludes the possibility that the excess of β_2m in the C282Y mutants (due to the inability of HFE to complex with β_2m) forces the premature MHC class I assembly. After incubation at 37°C, the class I complexes recovered with W6/32 appeared with different kinetics from the complexes isolated from lysates kept at 0°C (Figure 5A). In C282Y mutant cells class I heterotrimers were present in the initial chase times while in control cells class I complexes could be isolated only after 2 to 4 min (Figure 5A, W6/32-37°C). For quantitative comparison the W6/32 precipitated products shown in Figure 5A were scanned with a phosphoimager. The percentage of peptide loaded MHC class I complexes

present at each chase time was set as the ratio between the amount of W6/32 recovered class I complexes after the 37°C incubation and the amount recovered from the lysates kept at 0°C. Analysis of the data obtained revealed that in C282Y mutant cells the percentage of class I heterotrimers was significantly higher than in control cells. In C282Y mutant cells approximately 40% of the class I molecules recognized by W6/32 were in the heterotrimer conformation at the beginning of the chase, while only after 5 to 6 min of chase a similar amount of peptide loaded class I molecules was observed in control cells (Figure 5B).

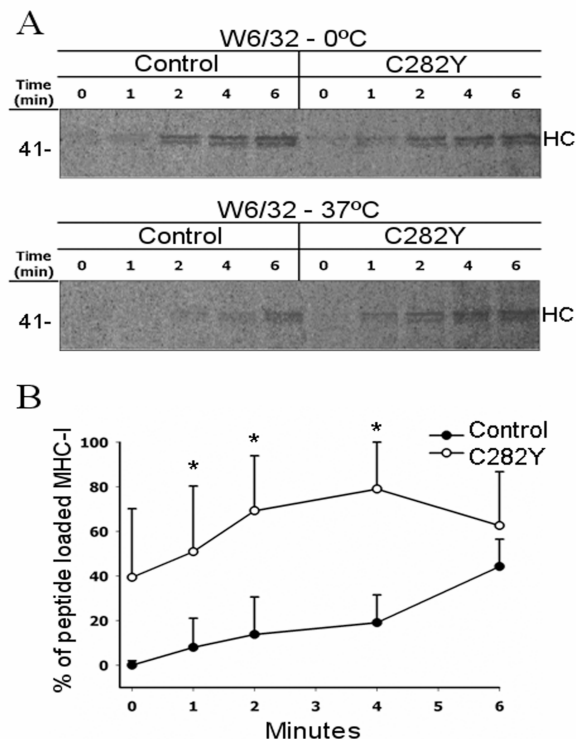


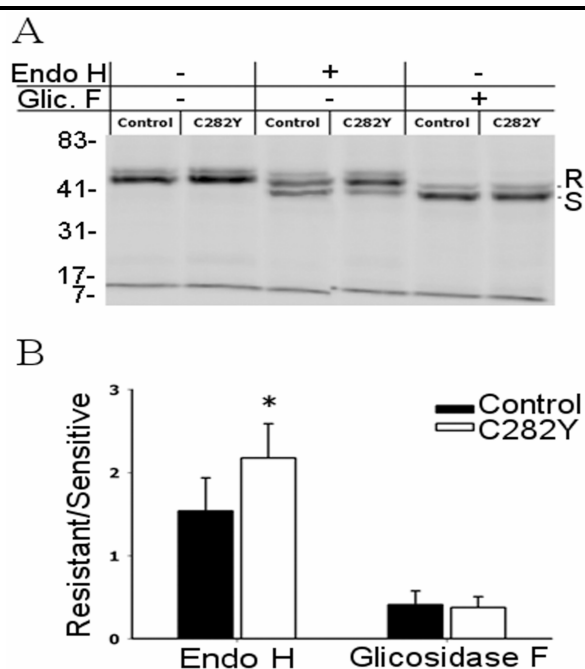
Figure 5 - Peptide loading is faster in C282Y homozygous cells. (A) PBMC without the C282Y mutation (Control) or C282Y homozygous (C282Y) were ^{35}S labeled during 5 minutes and chased for the indicated times. Half of the lysates was incubated at 37°C and the other half maintained at 0°C. MHC class I complexes were recovered with W6/32 mAb and subjected to 10% SDS-PAGE. Molecular weight markers are shown on the left. Data shown represent one individual experiment using PBMC from one WT blood donor and one C282Y homozygous HH patient. Five experiments with similar results were made. (B) Percentage of peptide loaded MHC class I molecules present at each chase time. Note the significant difference in the appearance kinetics of class I molecules when maintained at 0°C and after 37°C exposures. The asterisks indicate a statistically significant difference between the C282Y mutants and the controls ($p < 0.05$). Data are representative of 5 independent experiments (each performed with PBMC from one WT control blood donor and one C282Y homozygous HH patient).

ER egress of MHC class I molecules is faster in C282Y mutant PBMC

The previous results revealed a decreased MHC class I surface expression in C282Y mutant cells. In addition, the peptide loading occurs faster than in WT PBMC. Then, this should be reflected in the rate of ER maturation of class I molecules. To analyze if the ER retention time for MHC class I complexes differed between C282Y mutant and WT cells, but also to determine whether diminished MHC class I cell surface expression in mutant PBMC was related to the rate of maturation or the transport of class I heterotrimers, immunoprecipitation experiments with the W6/32 mAb were done using ^{35}S -met/cys labeled cells. Only WT (Control) and C282Y homozygous PBMC were used in these experiments. Intracellular processing of MHC class I N-linked glycans and ER egress can be estimated from the conversion of immature HC to mature Endo H resistant molecules. Analysis of the data obtained revealed that after a 2.5 hours biosynthetic labeling, the majority of MHC class I molecules acquired Endo H resistance in C282Y homozygous cells (as indicated by the stronger intensity of the

band with an apparent higher molecular weight, corresponding to the glycosylated form of the complex). In control cells only about one half of the complexes are Endo H resistant. No differences were observed in the Glycosidase F digested complexes suggesting that the reaction occurred with the same efficiency (Figure 6A). The W6/32 precipitated complexes treated with either Endo H or Glycosidase F, were scanned with a phosphoimager and the results plotted as the ratio between the intensity of the glycosidase-resistant over the -sensitive form. When compared to controls, C282Y homozygous cells showed a statistically significant higher proportion of Endo H resistant MHC class I molecules (Figure 6B) indicating that in the mutant cells the ER egress of class I molecules occurs faster than in controls.

Figure 6 - ER egress of class I molecules is faster in C282Y mutants. (A) After 2.5 hours ^{35}S metabolic labeling, W6/32 immunoprecipitates were digested for 6 hours at 37°C with Endo H, n-Glycosidase F or mock treated and separated by 10% SDS-PAGE. The position of the Resistant and Sensitive forms of MHC class I HC is indicated (R and S, respectively). Molecular weight markers are shown on the left. (B) Digestion resistant and sensitive class I forms were quantified by phosphoimaging and are presented as R/S ratio. The asterisk indicates a statistically significant difference between the C282Y mutants and the control cells ($p < 0.05$). Data are representative of five independent experiments, each performed with cells from one WT control blood donor and one C282Y homozygous HH patient.



Reduced peptide stability of MHC class I molecules in C282Y homozygous cells

To investigate the thermostability of the MHC class I molecules (which is strictly related to the affinity of the bound peptide) cell lysates were incubated for 3 hours at 0°, 25°, 37° and 42°C and the class I complexes recovered with W6/32. As shown by the variation of the band intensity (Figure 7A), MHC class I molecules from C282Y homozygous cells are more sensitive to increasing temperatures. At 0° and 25°C, C282Y mutant PBMC have more assembled class I complexes. After incubation at 37°C the amount of recovered class I molecules decreases more than 70%, approximately to the same level of the WT cells which show a 46% decrease. At 42°C only a small fraction of the assembled MHC molecules resist in the mutant cells.

Low temperatures (25°C) allow the cell surface expression of class I HC/ $\beta_2\text{m}$ heterodimers without peptides (Ljunggren et al. 1990). Compared to 37°C, overnight culture of C282Y and WT PBMC at 25°C revealed a 16% increase of the MHC class I surface expression in the mutant cells, while WT PBMC showed only a 7% increase (Figure 7B).

To investigate the surface stability of the MHC class I complexes, WT and C282Y homozygous PBMC were treated with 20 $\mu\text{g/ml}$ of brefeldin A (BFA) (Yewdell and Bennink 1989). The mean fluorescence intensity (MFI) of the W6/32 staining before and after 5 hours culture in the presence of BFA was compared (Figure 7C). In WT PBMC the BFA treatment reduced the MFI by 11% while in C282Y cells the MFI was reduced by 20%, indicating a faster turnover of cell surface MHC class I molecules in the mutant cells.

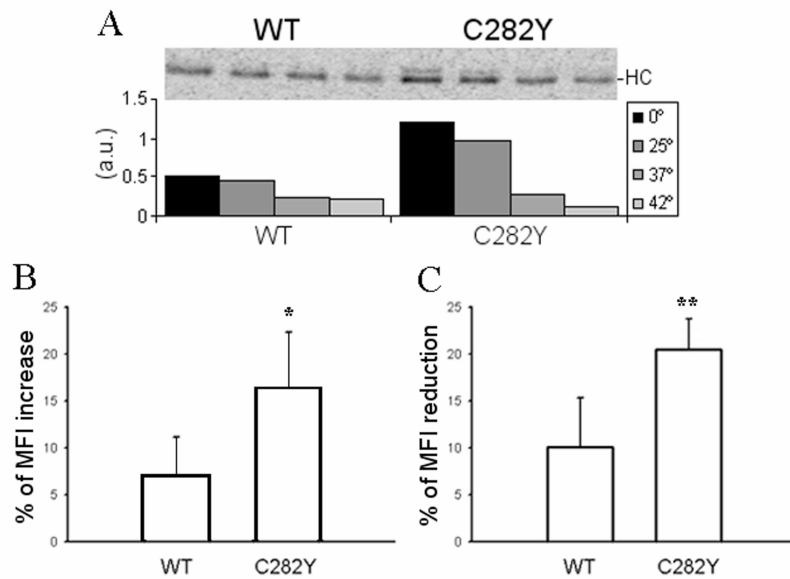


Figure 7 – Peptide stability and cell surface turnover of MHC class I molecules. (A) After 2.5 hours ^{35}S labeling, lysates from 10×10^6 cells were divided in four equal fractions and incubated at 0, 25, 37 and 42°C during 3 hours before W6/32 immunoprecipitation. The bars in the graphic represent the intensity of each single band determined by phosphorimaging (a.u. = arbitrary units). Representative data from one experiment repeated four times in a total of 4 WT and 7 C282Y homozygous subjects with similar results. (B) PBMC were cultured overnight at 25°C and 37°C before W6/32 surface staining. Results are presented as percentage of MFI increase of the cells cultured at 25°C.

The asterisk indicates a statistically significant difference between the C282Y and the WT cells ($p < 0.05$). Data compare 6 C282Y homozygous to 4 WT subjects. (C) W6/32 surface staining was performed before and after culturing WT and C282Y homozygous PBMC for 5 hours in the presence of 20 $\mu\text{g/ml}$ Brefeldin A. Results are presented as percentage of MFI reduction after the Brefeldin A treatment. The asterisks indicate a statistically significant difference between the C282Y and the WT cells ($p < 0.01$). Data compare 8 C282Y homozygous to 6 WT subjects.

Discussion

Interest in the HFE C282Y mutation in HH has focused on the role of HFE in iron metabolism. Although earlier reports identified anomalies of T cell subsets (Reimao et al. 1991; Porto et al. 1997) and of the CD8+ T cell receptor repertoire in C282Y carriers (Cardoso et al. 2001), no direct HFE role in immunological molecular mechanisms was investigated so far. The present results constitute the first evidence for an impact of the C282Y HFE mutation in the MHC class I antigen presentation pathway. All experiments were done using *ex vivo* PBMC from healthy blood donors or HH patients at different stages of the phlebotomy treatment (supplementary data – Table S1). This underlines the biological significance of the reported findings and separates them from the role of HFE in cellular iron metabolism. In addition, since no significant differences were observed in a series of experiments performed using PBMC from the same HH patient throughout the phlebotomies (data not shown) it is possible to exclude a possible effect of the repeated bleeding on the previous results. Despite the expected variability among the different subjects studied, the results were highly reproducible excluding differences due to highly selected MHC class I alleles, that are known to behave differently with respect to assembly, class I loading and ER egress (Rein et al. 1987; Neefjes et al. 1993b). This was further confirmed by the analysis of the class I haplotype of the subjects studied (supplementary data – Table S2).

We have observed that C282Y mutant PBMC obtained from HH patients have decreased properly assembled MHC class I molecules surface expression in contrast to the increased levels of free class I HC present on the membrane of these cells. The specificity of these observations is supported by the inexistence of CD4 and CD8 surface expression variation between control and mutant cells. Analyzing the intracellular folding of the class I molecules, we found altered levels of both properly β_2m assembled and free MHC class I HC. While the C282Y mutants exhibited higher amounts of assembled class I molecules, the WT cells compensate this by a marked excess of free MHC class I HC. The data from the HLA-DR experiments revealed no differences between the WT and the mutant cells. The impact of the C282Y mutation on the MHC class I intracellular trafficking is substantiated by the quantitative dependency of the HFE allele (C282Y homozygous or C282Y heterozygous vs controls) observed in the assembly of MHC class I molecules.

We show also that in mutant cells MHC class I molecules acquire Endo H resistance earlier indicating that the ER egress occurs faster than in WT cells. The same happens with the peptide loading. We demonstrated that in mutant cells the assembly of class I heterotrimers takes place sooner than in control cells. Previous studies using BFA in cells containing a mutant form of MHC class I molecule (T134K) that fails to bind to TAP showed that the time spent by MHC class I complexes in the ER determines the extent of the peptide loading optimization (Lewis and Elliott 1998). The faster peptide loading and ER egress reported here may *per se* raise some questions about the stability of the class I heterotrimers formed in the C282Y mutant cells. The premature ER release of MHC class I molecules suggests that an incomplete peptide optimization takes place in C282Y mutant cells, which may account for the diminished stability and surface expression of MHC class I complexes. In fact, we show here that in C282Y mutants the peptide stability is lower and the turnover of surface expressed β_2m assembled class I HC is enhanced compared to that observed in the WT PBMC. Apparently this is due to a

premature dissociation of these molecules. Together with the lower β_2m assembled and higher free MHC class I HC levels observed at the cell membrane of mutant cells, this result indicates that the extra pool of surface expressed free class I HC in the C282Y PBMC results from the class I heterotrimers following peptide and β_2m dissociation.

All the findings reported in this study allow us to envisage some plausible models that could explain the abnormalities seen in the MHC class I pathway of C282Y mutant cells. In the first one, HFE itself would play a direct role in the maturation and quality control of MHC class I molecules. The interaction between HLA-DM and the MHC class II pathway constitutes a precedent of a well-studied example of peptide editing performed by a non-classical MHC molecule (Brocke et al. 2002). Viral subversion of the immune system involves the degradation or down-regulation of key proteins that participate in the antigen presentation pathways. While most viral proteins specifically target MHC class I molecules, the HCMV glycoprotein US2 was also shown to target HFE for proteasomal mediated degradation (Ben-Arieh et al. 2001; Vahdati-Ben Arieh et al. 2003). If HFE is involved in MHC class I maturation and peptide loading, this may explain why it deserves virus attention.

A second potential explanation excludes a direct role exerted by HFE attributing the cause of the anomalies seen in MHC class I to the availability of molecular chaperones caused by the presence of the C282Y mutated HFE. The impairment of HFE correct folding, due to this mutation, could lead to a change in the levels of occupancy of molecular chaperones like calreticulin, calnexin or ERp57. The mutation could also trigger the production of specific chaperones, like heat shock proteins, that act to ensure the correct folding or, ultimately, proteasomal degradation of accumulated misfolded products. Being involved in MHC class I assembly, an altered degree of availability of these molecules would influence the intracellular state of class I molecules.

While MHC class I molecules are present in all cell types, the HFE expression pattern is restricted. The antigen presenting cells (APC), monocytes and macrophages (including Kupffer cells) (Cardoso et al. 2004) are among the cells where HFE can be found. Unless another protein exists that could perform a similar role, the presumable function exerted by HFE may constitute a unique feature of the antigen presentation in these cells. We could speculate that APC have developed specific attributes to improve the efficiency of antigen presentation in which HFE could be implicated. This will be tested with selected APC.

The insufficient signaling by MHC class I-specific inhibitory receptors present on NK cells leads to activation and cytotoxicity against cells that are either MHC class I negative or deficient (Biaassoni et al. 2001). The reported observation that C282Y mutants have decreased cell surface expression of MHC class I molecules makes these cells suitable candidates for targeting by NK cells. A recent study revealed that down-regulation of MHC class I on resting lymphocytes was sufficient to make them susceptible to NK cell killing (Oberg et al. 2004). Besides, as MHC class I dictates the immune response of CD8+ CTL the reduction in the surface expression in C282Y mutant cells should result in a decreased CD8+ CTL activity. This has been shown previously in HH patients (Arosa et al. 1997).

Our findings add a novel component to the complex pathway of antigen presentation, indicating that the C282Y HFE mutation, in addition to its effects in iron homeostasis, is associated with abnormal expression of MHC class I molecules and impaired class I antigen presentation pathway.

Supplementary data

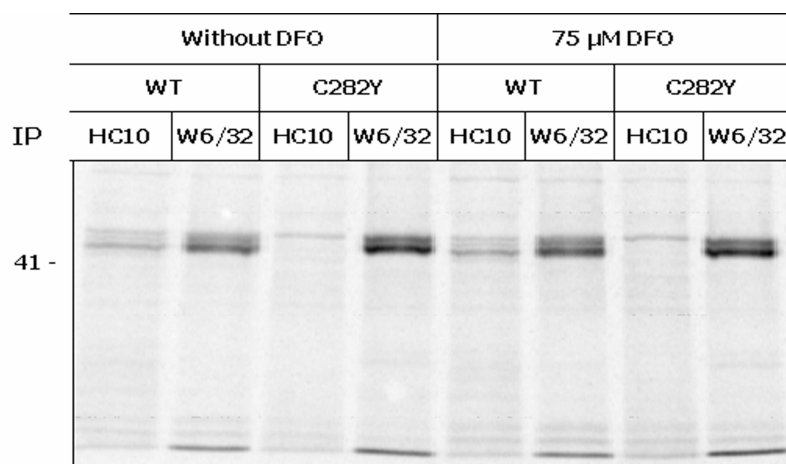


Figure S1 - PBMCs with (C282Y) or without (WT) the C282Y mutation were ³⁵S-labeled for 2.5 hours with or without 75 μM DFO, and class-I molecules were recovered with HC-10 and W6/32 mAbs and subjected to 10% SDS-PAGE. Molecular-weight markers are shown on the left. One representative experiment using PBMCs from one WT blood donor and one C282Y-homozygous HH patient is shown.

Table S1 – All the HH patients studied were grouped according to the phlebotomy treatment stage in which they were when the blood was collected for PBMCs isolation. IT = Intensive Treatment.

Phlebotomy stage	% of patients on treatment
Beginning of the IT	3.6
Middle of the IT	35
End of the IT	8.4
Maintenance	53

Table S2 – HLA-A, B and C allele frequency within the 48 HH patients studied.

HLA-A	Frequency	HLA-B	Frequency	HLA-C	Frequency
1	0.155	7	0.200	2	0.151
2	0.240	8	0.0625	3	0.0411
3	0.219	12	0.0125	4	0.260
11	0.0104	14	0.0250	5	0.0274
23	0.0104	15	0.0625	6	0.110
24	0.0730	18	0.0875	7	0.356
25	0.0208	27	0.0125	8	0.0136
26	0.0313	35	0.188	12	0.0136
29	0.0521	37	0.0875	15	0.0136
30	0.0208	38	0.0125		
31	0.0420	40	0.0250		
32	0.0104	44	0.100		
33	0.0104	49	0.0125		
68	0.0104	50	0.0125		
		51	0.0375		
		55	0.0125		
		57	0.0375		
		60	0.0125		

CHAPTER 3

STIMULATION OF AN UNFOLDED PROTEIN RESPONSE IMPAIRS MHC CLASS I EXPRESSION

de Almeida SF, Fleming JV, Azevedo JE, Carmo-Fonseca M, de Sousa M. *J Immunol.* 2007 Mar
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Abstract

HFE C282Y is an example of a mutant protein that does not fold correctly, is retained in the endoplasmic reticulum and was found previously to diminish surface expression of major histocompatibility complex class I (MHC-I). We now show that its expression in 293T cells triggers an unfolded protein response (UPR) as revealed by the increased levels of BiP, GRP94 and CHOP. Elevated levels of these proteins were also found in HFE C282Y homozygous peripheral blood mononuclear cells (PBMCs). Following the UPR induction a decrease in MHC-I cell surface expression was observed. This defect in MHC-I could be mimicked, however, by over-expression of transcriptionally active isoforms of ATF6 and XBP-1 that induces the UPR, and reversed in HFE C282Y expressing cells by using dominant negative constructs that block UPR signaling. The present results provide evidence to the finding that stimulation of an UPR affects MHC-I expression.

Introduction

Hereditary Hemochromatosis (HH) is an inherited autosomic recessive disease of iron metabolism characterized by an increased intestinal iron absorption that leads to iron overload of parenchymal cells in several organs (Cox and Lord 1989). HFE is a 343 residue type I transmembrane glycoprotein homologous to major histocompatibility complex class I (MHC-I) proteins (HFE is a member of the “non-classical MHC-I molecules” family). Early during its intracellular trafficking, HFE interacts with β_2 -microglobulin (β_2m) and only this heterodimer (HFE/ β_2m) is able to travel through the standard secretory pathway towards the cell surface (Feder et al. 1996). The observation that HFE binds to transferrin receptor I (TfR1), a homodimeric cell surface glycoprotein, which acts as the receptor for iron-loaded transferrin, implicated HFE in the regulation of iron homeostasis (Feder et al. 1998). The majority of HH patients carry an HFE mutation, in which cysteine at position 282 is changed to tyrosine (C282Y) (Feder et al. 1996). This mutation prevents the formation of an intramolecular disulfide bridge in the $\alpha 3$ domain of HFE abrogating β_2m association and inhibiting the trafficking of protein towards the cell surface (Feder et al. 1997). The C282Y mutant protein is retained in the endoplasmic reticulum (ER) and middle Golgi compartment, fails to undergo late Golgi processing and is subjected to accelerated degradation (Waheed et al. 1997).

Accumulation of unfolded or misfolded proteins in the ER activates specific signaling pathways that are collectively referred to as the unfolded protein response (UPR). These pathways act to alleviate stress by enhancing the folding and/or degradation of misfolded proteins, and by reducing the rate of protein synthesis (Schroder and Kaufman 2005c). BiP (also termed GRP78), an ER chaperone protein, is a key UPR sensor. In the absence of stress conditions BiP binds to a number of ER-stress transducing proteins, double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring kinase 1 (IRE1). This interaction keeps them inactive until the accumulation of misfolded proteins sequesters BiP and leads to the release and activation of the ER-stress transducers (Bertolotti et al. 2000; Zhang and Kaufman 2004). Activation of PERK results in the phosphorylation of proteins that regulate protein translation and UPR signaling (Cullinan et al. 2003). ATF6 is a transcription factor that becomes cleaved and relocates to the nucleus where it promotes expression of UPR responsive genes (Haze et al. 1999; Shen et al. 2002). IRE1 proteins are ribonucleases that mediate the alternative splicing of XBP-1 mRNA to produce an active transcription factor that likewise promotes transcription of UPR responsive genes (Yoshida et al. 2001).

The UPR activation has several important physiological implications (Schroder and Kaufman 2005c). Of relevance here are recent studies investigating whether MHC-I degradation caused by Human Cytomegalovirus (HCMV) proteins (US11 and US2) is sensitive to whether or not a cell is experiencing UPR stress (Tirosh et al. 2005; Hegde et al. 2006). MHC-I molecules are cell surface glycoproteins expressed in virtually all nucleated mammalian cell types. MHC-I molecules present antigens to CD8+ T cells. These antigens are mainly derived from the proteolysis of cytosolic proteins, including those that are normally derived from infecting viruses (Rock and Goldberg 1999; Reits et al. 2000; Schubert et al. 2000). The resulting peptides are translocated into the ER lumen by the TAP1/TAP2 heterodimer (Androlewicz et al. 1993; Neefjes et al. 1993a), where they are further

processed by the ER associated aminopeptidase (ERAAP) (Saric et al. 2002; Serwold et al. 2002). The formation of the peptide loading complex (PLC) facilitates the binding of peptide to MHC-I/ β_2m heterodimers (Cresswell et al. 1999). At this stage, a set of quality control mechanisms acts to ensure that high affinity peptides are loaded onto class I molecules (Elliott and Williams 2005). The newly formed peptide loaded MHC-I molecules are then released from the PLC and delivered to the cell surface via the standard secretory pathway.

We have recently shown that the C282Y HFE mutation interferes with MHC-I peptide loading and optimization in the ER and that cells carrying the mutant protein display reduced cell surface expression of MHC-I molecules (de Almeida et al. 2005). Given that misfolding ER proteins are capable of activating the UPR and the recent studies on the virally induced UPR and MHC-I processing (Tirosh et al. 2005; Hegde et al. 2006), we hypothesized that MHC-I anomalies observed in HFE C282Y PBMCs might arise as a result of an ER stress associated with the mutant HFE proteins. Here we test this hypothesis and demonstrate that an active UPR associated with the HFE C282Y mutant protein does indeed impair MHC-I cell surface expression.

Material and Methods

Antibodies and plasmids The following Abs were used: The mAb W6/32 (Dako, Glostrup, Denmark) recognizes MHC-I molecules; HC10 (Mouse anti-Human MHC-I) recognizes free MHC-I heavy chains (a kind gift from Dr Jacques Neefjes, The Netherlands Cancer Institute, Amsterdam); 8C-10 (Mouse anti-human HFE) recognizes HFE/ β_2m heterodimers (a kind gift from Dr Rachel Ehrlich, Department of Cell Research and Immunology, Tel Aviv University, Israel); rabbit anti-HFE cytoplasmic tail (CT); (Ben-Arieh et al. 2001) mouse anti-KDEL (detects BiP and GRP94) and rabbit anti- β -Actin (Abcam, Cambridge, UK); donkey-anti-Mouse-FITC conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA); FITC-anti-CD71 (TfR1) (BD Pharmingen, San Jose, CA); mouse anti-E-Cadherin (Immunotech, Marseille, France).

The HFEwt-pcDNA3 construct was a kind gift from Dr Luísa Salter-Cid. HLA-A2-pcDNA3.1 and β_2m -pcDNA3.1 constructs were a kind gift from Dr Hal Drakesmith (University of Oxford, UK). CMV driven ATF6-DN (Wang et al. 2000) and XBP-1-DN (Lee et al. 2003) plasmids were gifted by Dr Ron Prywes (Columbia University, New York, NY) and Dr Laurie Glimcher (Harvard School of Public Health, Boston, MA) respectively. The pEP7-nATF6-FL vector expressing a nuclear targeted and transcriptionally active fragment of ATF6 (amino acids 1-373) was generated by *pfu* (Stratagene, La Jolla, CA) PCR amplification using HEK293 cell cDNA as template and specific sense (GGGAAGCTTGCCACCATGGGGGAGCCGGCTGGGGTTGCCGGCACCATGGAG) and antisense (CCCCCTCGTCGACGGGACTTTAAGCCTCTGGTTCTCTGACACAACCTTC) primers. PCR products were cloned into the *HindIII* and *Sall* sites of the FLAG tagged pEP7-FL vector as described previously (Fleming et al. 2004). A plasmid encoding the spliced form of XBP-1 was kindly gifted by Dr K. Mori (Kyoto University, Japan) and acted as template for a *pfu* PCR amplification using the specific sense (GGGAAGCTTGCCACCATGGTGGTGGTGGCAGCCGCGCCGAAC) and antisense (CCCCCTCGTCGACACACTAATCAGCTGGGGAAAGAGTTCATTGGC) primers. The amplified product was cloned into the *HindIII* and *Sall* sites of the pEP7-FL vector to give the pEP7-sXBP-1-FL vector.

Cells Peripheral blood mononuclear cells (PBMCs) were obtained from healthy blood donors (wild-type HFE) and hereditary hemochromatosis patients (HFE C282Y homozygous) at the HH Clinic, Santo António General Hospital (Porto, Portugal), after informed consent was obtained, according to the declaration of Helsinki. The study protocol was approved by the hospital's ethical board. When possible, in each individual experiment subjects were matched by sex and age. PBMCs were isolated following an established procedure. Briefly, after centrifugation over Lymphoprep (Nycomed, Oslo, Norway), PBMCs were washed with HBSS. Contaminating RBCs were lysed in 10 mM Tris, 150 mM NH_4Cl , pH 7.4 at 37°C for 10 minutes. Human Embryonic Kidney 293T cells were obtained from the American Type Culture Collection.

HFE genotyping HFE genotyping was done using the Haemochromatosis StripAssay^B kit (ViennaLab, Vienna, Austria) according to the manufacturer's protocol.

Site-directed Mutagenesis The QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA) was used as described in manufacturer's protocol to produce the HFE C282Y and the HLA-A2 C283Y constructs. Mutagenesis was confirmed by DNA sequencing of both strands.

Transfections For the RT-PCR, flow cytometry and metabolic labeling experiments, 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 60 mm plates accordingly to the manufacturer's protocol. At the time of transfection, cells were 90-95% confluent. Opti-MEM was used to dilute both DNA and Lipofectamine at a final DNA:Lipofectamine ratio of 1:2.5. After transfection cells were incubated for 48 hours in DMEM w/ GlutaMAX medium (Gibco, Grand Island, NY) containing 1% of penicillin/streptomycin/amphotericin solution (Sigma-Aldrich, St. Louis, MO) and 10% heat-inactivated fetal bovine serum.

RT-PCR Total RNA was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For analysis of actin, BiP, CHOP and HFE gene expression, cDNA was reverse transcribed and amplified from 100ng of total RNA using Access RT-PCR (Promega, Madison, WI) following manufacturer's instructions. After separation on 1.5% agarose gels with ethidium bromide (Sigma-Aldrich, St. Louis, MO), the specific bands were quantified with ImageQuant v5.1 software. The primer sequences used for the amplifications were:

BiP 5'-CCTGGGTGGCGGAACCTTCGATGTG-3' and

5'-CTGGACGGGCTTCATAGTAGACCGG-3';

CHOP 5'-GCCTTTCTCCTTTGGGACACTGTCCAGC-3' and

5'-CTCGGCGAGTCGCCTCTACTTCCC-3';

HFE 5'-ATGGGCCCGCGAGCCAGGCCGGCGCTTCT-3' and

5'- TCACTCACGTTTACGCTAAGACGTAGTGCC-3'

For analysis of XBP-1 splicing 5µg of RNA were reverse-transcribed using ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) following manufacturer's instructions. PCR amplification was performed with the DFS-Taq DNA polymerase (Bioron GmbH, Ludwigshafen, Germany) using a previously described pair of primers that differentiate between the spliced and unspliced forms of XBP-1 (Yoshida et al. 2001). Amplified products were resolved on 2% agarose gels. Pharmacological induction of the UPR was performed by incubating the cells with 2mM DTT (Sigma-Aldrich, St. Louis, MO) for 5 hours (Kaneko et al. 2002).

Quantitative Real Time RT-PCR For Real-Time PCR analysis, total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Following treatment with 2U/sample of RQ1 DNase, in the presence of 50 U/sample of RNase inhibitor (Invitrogen), for 30 min at 37°C, 1 µg of RNA were reverse-transcribed, using ThermoScript reverse transcriptase (Invitrogen), following the manufacturer's instructions. Expression of MHC-I (HLA-A) was evaluated by quantitative Real-Time PCR (qRT-PCR) with the ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA, USA). qRT-PCR was

conducted in triplicates, using 1x SYBR Green PCR Master Mix (Applied Biosystems). Quantification of β -Actin gene expression was performed as a control. Relative expression levels were calculated as $2^{-(Ct \text{ human } \beta\text{-Actin} - Ct \text{ HLA-A} \times 10000)}$ (for details see ABI PRISM 7700 - User Bulletin #2). MHC-I (HLA-A allele) primer sequences are: 5'-CACGAGCTCCGTGTCCTGGGTCTG-3' and 5'-AAGGATTACATCGCCCTGAACGAGGAC-3'.

Flow cytometry 48 hours after transfection, 293T cells were stained for surface HFE, TfR, E-Cadherin and MHC-I. Briefly, 5×10^5 cells were washed in ice cold PBS, 0.2% BSA, 0.1% NaN₃ followed by incubation at 4°C with a saturating amount of primary Ab for 30 minutes in 96-well plates. After 3 washes cells were incubated with secondary Ab for 30 minutes on ice. Cells were washed twice and flow cytometry analysis performed in a FACScalibur. For each sample a minimum of 10 000 events were acquired. To define the background staining, irrelevant mAbs of the same isotype were used. We frequently observed two histogram peaks in the experiments which may reflect the existence of two populations of cells: transfected and non-transfected.

Metabolic labeling 48 hours after transfection, 2.5×10^6 293T cells were starved for 1 hour in Cysteine/Methionine free DMEM medium (Gibco, Grand Island, NY) supplemented with 1% L-glutamine and labeled for 3 hours with 140 μ Ci/ml Pro-Mix L-[³⁵S]Cysteine/Methionine (Amersham Biosciences UK, England). After labeling, cells were washed three times with HBSS and lysed in ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Triton-X, Complete EDTA Free-protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 10 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO). The nuclei and cell debris were removed by centrifugation and the lysates pre-cleared with 100 μ l protein-A Sepharose beads slurry (50%) for 1 hour at 4°C. The lysates containing equivalent amounts of TCA-precipitable radioactivity were incubated at 4°C with anti-KDEL. Immunocomplexes were pulled down with protein-A sepharose beads and washed three times in ice-cold lysis buffer. After addition of gel loading buffer solution (Laemmli 1970) with 10% β -mercaptoethanol and boiling for 5 minutes, samples were loaded on 10% SDS-PAGE.

SDS-PAGE and Quantitation 10% SDS-PAGE was performed using a Bio-Rad Mini Protean II kit. Gels were fixed in 10% acetic acid, 40% methanol, incubated for 30 minutes with Amplify solution (Amersham Biosciences UK, England) dried and exposed to a radioactivity storage screen. Quantitation was performed using a Typhoon phosphorimager (Molecular Dynamics, Sunnyvale, CA) with ImageQuant v5.1 software.

Western Blot Total protein concentration from whole cell lysates was determined with RC/DC Protein Assay (Bio-Rad, Hercules, CA) and 30 μ g (PBMC) or 20 μ g (293T transfected cells) separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose Hybond-C membrane (Amersham Biosciences UK, England). After blocking at 4°C with 5% dry milk/0.05% Tween 20 in PBS (PBS-T), the membrane was incubated with anti-HFE (CT), anti-KDEL or HC10, washed 3X with PBS-T and detected with the respective HRP conjugated secondary antibody (Molecular Probes, Eugene, OR) and an enhanced chemiluminescence substrate (Pierce,

Rockford, IL). As a loading control, the membrane was stripped using Restore WB Stripping Buffer (Pierce, Rockford, IL) and incubated with anti- β -Actin.

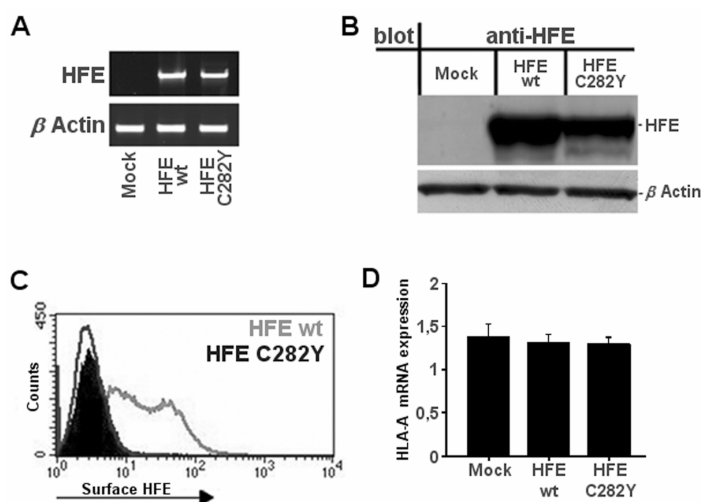
Statistical analysis To test the significance of the differences observed the Student's T-test was used. In all tests the statistical significance was 2-sided and considered at $P < 0.05$. Data are displayed as mean $\pm$ 1SD.

Results

The cell line model system

We have recently demonstrated that PBMCs derived from HFE C282Y homozygous patients have decreased MHC-I cell surface expression (de Almeida et al. 2005). To help explain the molecular and cellular mechanisms linking this phenotype with mutant HFE we developed a cell line model for use in parallel with experiments on human PBMCs. The 293T cell line was chosen because it does not produce endogenous HFE and, as all nucleated cells, expresses MHC-I (Figure 1A, B and D). Western blot analysis was used to demonstrate expression of the HFE wt and C282Y proteins in transient transfection experiments, and flow cytometry used to confirm previous reports (Feder et al. 1997; Waheed et al. 1997) that the C282Y mutant is unable to travel to the cell surface (Figure 1B and C). Most importantly, however, qRT-PCR showed that MHC-I mRNA levels were unaffected by expression of either the wt or mutant HFE proteins (Figure 1D). In these studies, and unless otherwise stated, a plasmid encoding β_2m was co-transfected with HFE expression vectors in order to ensure that sufficient quantity of this protein was available for HFE's correct assembly.

Figure 1 – HFE and MHC-I expression in the 293T cell line model (A) HFE mRNA levels were analyzed by RT-PCR of total RNA in 293T cells co-transfected with β_2m plus HFE wt, HFE C282Y or the empty vector (Mock). As a control, β -Actin RT-PCR was also performed. (B) HFE from whole cell lysates of Mock/ β_2m , HFE wt/ β_2m and HFE C282Y/ β_2m overexpressing 293T cells was detected by Western blot using the anti-HFE (CT) antibody. As a loading control, the membrane was striped and incubated with anti- β -Actin. Results shown are representative of three independent experiments. (C) HFE cell surface expression in HFE wt/ β_2m and C282Y/ β_2m co-transfected cells was assessed by flow cytometry after staining with 8C10 anti-HFE Ab and anti-HFE CT Ab respectively. The grey and black lines in the histogram represent the cell surface expression of HFE wt and HFE C282Y respectively, 48 hours after each transfection. The filled area in the histogram represents the negative control obtained by incubation with an isotype-matched non-specific antibody. Results are representative of three independent experiments. (D) MHC-I (HLA-A allele) mRNA levels of 293T cells co-transfected with β_2m plus HFE wt, HFE C282Y or the empty vector (Mock) were assessed by qRT-PCR. The graph represents the average $\pm$ 1SD of three independent experiments. β -Actin was used as an endogenous control gene.



The HFE C282Y mutant protein induces the UPR activation

To determine whether the UPR is activated in cells expressing the C282Y mutant form of HFE, the mRNA and/or protein levels of three UPR targets, BiP and GRP94 (two ER-resident chaperones) and C/EBP homologous protein (CHOP), were assessed. Increased levels of both BiP and CHOP gene transcripts were found by RT-PCR in 293T cells transiently co-transfected with HFE C282Y/ β_2m compared to mock treated and HFE wt/ β_2m expressing cells (Figure 2A). Forty-eight hours after HFE C282Y/ β_2m co-transfection there was a 2.9

fold increase in BiP and a 4.7 fold increase in CHOP gene transcript levels in relation to mock 293T cells. HFE wt/ β_2m co-transfection led to a 1.3 and a 1.4 fold increase in BiP and CHOP levels respectively. A similar and statistically significant ($P < 0.01$) result was found when PBMCs isolated from C282Y homozygous HH patients were compared to PBMCs from control groups (Figure 3A). BiP and CHOP mRNA levels were respectively 2.0 and 1.8 fold higher in C282Y homozygous PBMCs.

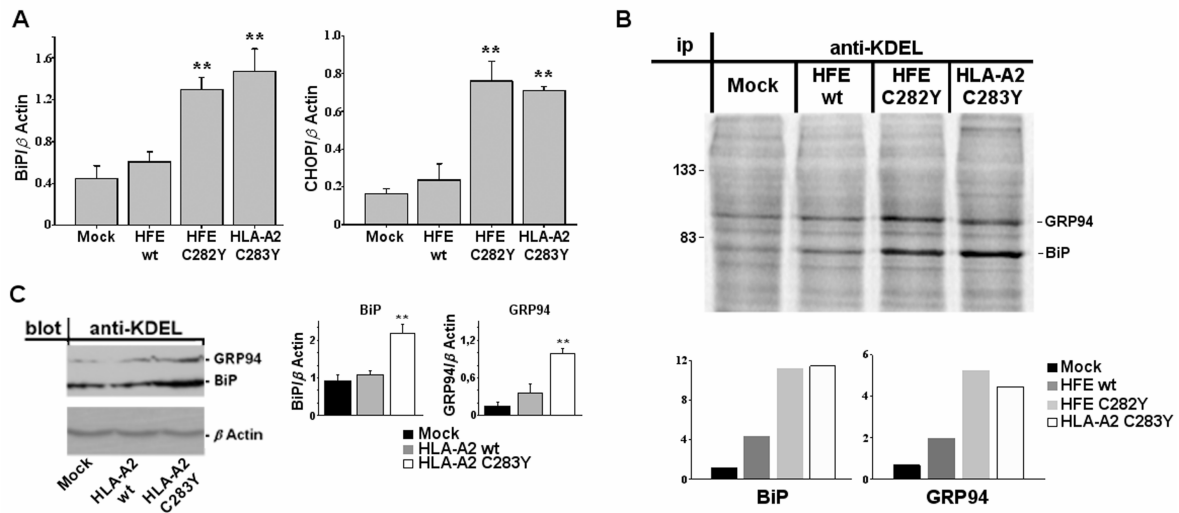
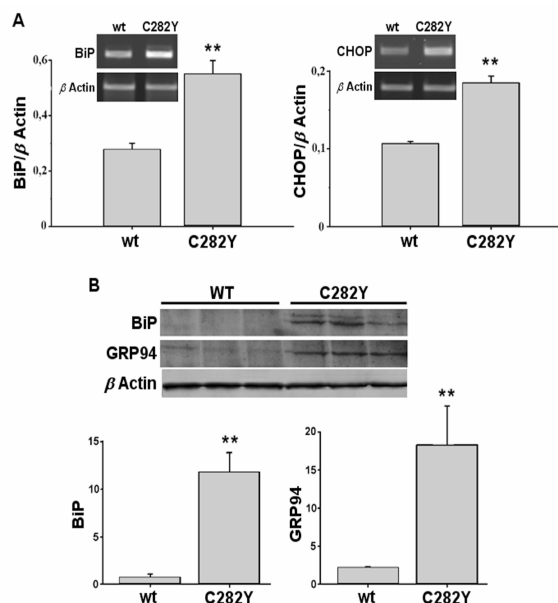


Figure 2 – Increased levels of UPR target genes are found in 293T cells expressing C282Y mutant HFE and MHC_I proteins (A) BiP and CHOP mRNA levels (normalized to β -Actin) from empty plasmid (Mock), HFE wt, HFE C282Y or HLA-A2 C283Y expressing 293T cells co-transfected with the β_2m expressing plasmid were assessed by semi-quantitative RT-PCR. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences ($P < 0.01$) compared to Mock cells. **(B)** 48 hours post co-transfection with β_2m plus HFE wt, HFE C282Y, HLA-A2 C283Y or the empty vector (Mock), 293T cells were biosynthetically labeled for 3 hours. BiP and GRP94 proteins were recovered with an anti-KDEL mAb and subjected to 10% SDS-PAGE. The position of both proteins in the gel is indicated. Molecular weight markers are shown on the left. The intensity of the bands corresponding to BiP and GRP94 was quantified by phosphorimaging and plotted on a bar graph with arbitrary units. Data shown represent one individual experiment of a set of four done with similar results. **(C)** To evaluate the UPR activation in 293T cells transfected to co-express HLA-A2 wt/ β_2m and HLA-A2 C283Y/ β_2m , BiP and GRP94 levels were detected by Western blot with anti-KDEL mAb. As a loading control, the membrane was striped and incubated with anti- β -Actin. The intensity of the bands corresponding to BiP and GRP94 was normalized to β -Actin levels and plotted on a bar graph as average + 1SD of three experiments. The asterisks represent statistically significant differences ($P < 0.01$) between the HLA-A2 C283Y and the Mock transfected cells.

As an independent measurement of UPR activation, BiP and GRP94 protein levels in transfected 293T cells and primary cultured PBMCs were also quantified. Assessment of protein levels in PBMCs was done by western blot analysis of total cell lysates with an anti-KDEL antibody. Significantly higher amounts of BiP and GRP94 were observed in C282Y-homozygous compared to wt PBMCs (Figure 3B). For 293T cells we performed metabolic labeling two days post-transfection and endogenous BiP and GRP94 were retrieved by immunoprecipitation. Again, significantly increased quantities of the two ER chaperones, BiP and GRP94, were found in the 293T HFE C282Y/ β_2m expressing cells when compared to HFE wt/ β_2m co-transfected or mock cells (Figure 2B).

Figure 3 – Primary cultured HFE C282Y mutant PBMCs have an active UPR (A) BiP and CHOP mRNA levels from wt or C282Y homozygous (C282Y) PBMCs were assessed by semi-quantitative RT-PCR. The graph represents the average from six HFE wt and six HFE C282Y homozygous PBMCs BiP and CHOP levels normalized to β -Actin. The asterisks represent statistically significant differences ($P < 0.01$) between the wt and the C282Y homozygous PBMCs. (B) BiP and GRP94 from whole cell lysates of wt or C282Y homozygous (C282Y) PBMCs were detected by Western blot with anti-KDEL. 30 μ g of total protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane probed with anti-KDEL and anti- β -Actin as a loading control. Results from PBMCs obtained from three different blood donors (HFE wt) and three different HH patients (C282Y homozygous) are shown. The intensity of the bands obtained in a total of six HFE wt and six HFE C282Y homozygous subjects was quantified and the results plotted as average $\pm$ 1SD. The asterisk represents statistically significant differences ($P < 0.01$) between the wt and the C282Y homozygous PBMCs.



While these studies demonstrated a specific C282Y-induced increase in the expression of UPR-related markers, we were interested also in examining the changes in UPR signaling that occur as a consequence of mutant HFE expression. To do this we looked for IRE1 mediated signaling as demonstrated by increased splicing of XBP-1. 293T cells were transfected with empty vector, vector expressing the C282Y HFE mutant, or vector expressing the spliced form of XBP-1 (sXBP-1) as a positive control. Mock transfected cells were cultured in the presence or absence of UPR-inducing DTT for 5 hours prior to harvest. Total RNA was extracted and RT-PCR performed using primers that differentiate between the unspliced and spliced forms of XBP-1 (19 base difference). These experiments clearly demonstrated that IRE1 signaling and alternative splicing of XBP-1 is occurring in the C282Y expressing cells. The combined data from these experiments suggested that the level of alternative splicing was similar to that associated with the pharmacological induction of UPR signaling by DTT (Figure 4).

The defect in the α 3 domain correct assembly is the stimulus for the UPR activation

To confirm that the UPR activation occurs as a result of the inability to correctly assemble the α 3 domain of HFE C282Y protein, the same point mutation was inserted in the HLA-A2 allele of MHC-I. HFE and MHC-I proteins are structurally very similar and share high aminoacid identity (Lebron et al. 1998). The cysteine at position 282 involved in the formation of an intra-molecular disulfide bridge in the α 3 domain of HFE (essential for β_2 m association) (Feder et al. 1996) is conserved in MHC-I (at position 283), playing the same role in the folding and β_2 m association of this protein. Thus, by inserting this mutation in MHC-I (HLA-A2 C283Y), a protein with the same folding defect (incorrect assembly of the α 3 domain) of HFE C282Y is produced. 293T cells transiently co-transfected with the HLA-A2 C283Y mutant expression vector and β_2 m exhibited BiP and CHOP mRNA as well as BiP and GRP94 protein levels comparable to those observed in the HFE C282Y transfected cells (Figure 2)

suggesting that the $\alpha 3$ domain misfolding is the stress stimulus that leads to the UPR activation. Unlike its mutant counterpart, overexpression of the wt MHC-I protein in 293T cells did not activate the UPR (Figure 2C).

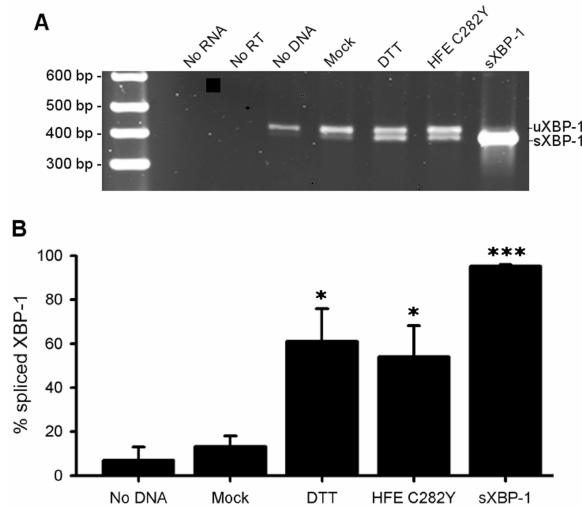


Figure 4 – HFE C282Y leads to IRE1 signaling and alternative splicing of XBP-1 (A) Total RNA from untransfected cells (No DNA) or cells transfected with empty plasmid in the absence (Mock) or presence (DTT) of 2mM DTT, HFE C282Y or sXBP-1, was amplified by RT-PCR. Amplified products were subjected to 2% agarose gel electrophoresis. First and second lanes (No RNA and No RT) show the RT-PCR negative controls. The position of the unspliced and spliced XBP-1 (uXBP-1 and sXBP-1 respectively) is shown on the right. The lengths of DNA size markers are shown on the left. **(B)** The percentage of spliced XBP-1 was determined by dividing the intensity of the sXBP-1 band in (A) for the total XBP-1 (unspliced + spliced). Data are from 3 independent experiments and represent the mean + 1SD. Asterisks indicate statistically significant differences comparatively to the Mock (* $P < 0.05$; *** $P < 0.001$).

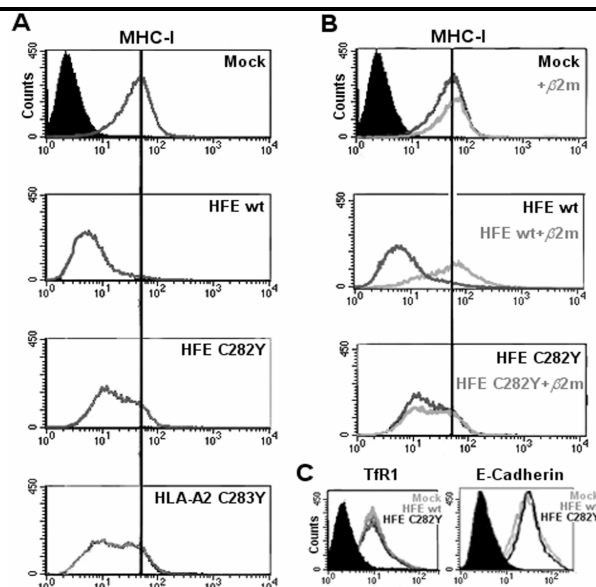
The MHC-I anomalies observed in HFE C282Y mutant cells are not due to limiting $\beta 2m$

We have previously demonstrated that PBMCs from HH patients expressing C282Y have a reduced cell surface expression of MHC-I.²⁴ We wondered whether C282Y expression in the transfected cell model was likewise associated with decreased surface expression of MHC-I. 293T cells were transfected to express the wild type or C282Y mutant forms of HFE in the presence or absence of supplemental (ie. co-transfected) $\beta 2m$. Figure 5A shows that the expression of either HFE wt or HFE C282Y in the absence of supplemented $\beta 2m$ resulted in decreased surface expression of MHC-I molecules compared to controls. This effect appears specific for surface expression of MHC-I as no differences were observed in surface expression of TfR1 and E-Cadherin (Figure 5C).

As HFE associates early during its intracellular trafficking with $\beta 2m$, the introduction of HFE wt would lead to competition for the available endogenous $\beta 2m$. The reduction of cell surface MHC-I in HFE wt expressing cells in Figure 5A could therefore be due to the sequestration of the free $\beta 2m$ pools by the overexpressed protein. This would not be a feature of HFE C282Y expression due to its inability to bind $\beta 2m$. To test this hypothesis, $\beta 2m$ was co-transfected with HFE wt or HFE C282Y in 293T cells. Flow cytometry analysis performed 48 hours post-transfection showed partial rescuing of MHC-I cell surface expression after $\beta 2m$ co-transfection with HFE wt, but not with HFE C282Y (Figure 5B).

These results appear to exclude $\beta 2m$ competition as an explanation for decreased surface expression of MHC-I, and instead appear to link protein misfolding with the observed MHC-I defects. Consistent with this we noted that expression of the UPR-inducing HLA-A2 C283Y mutant also decreased surface expression of MHC-I (Figure 5A).

Figure 5 – HFE C282Y overexpression decreases MHC-I cell surface expression independently of β_2m levels (A) 293T cells transfected with HFE wt, HFE C282Y, HLA-A2 C283Y or the empty vector (Mock) were surface stained for MHC-I and analyzed by flow cytometry. The lines in each histogram represent the MHC-I cell surface expression 48 hours after each transfection. **(B)** HFE wt and HFE C282Y expressing and Mock treated 293T cells were co-transfected with β_2m and surface stained for MHC-I. The grey lines represent the MHC-I surface expression in β_2m co-transfected cells and the black lines the cells that do not overexpress β_2m . **(C)** TfR1 and E-Cadherin cell surface expression was analyzed in HFE wt, HFE C282Y or Mock expressing 293T cells. The filled area in all histograms represents the negative control obtained by incubation with an isotype-matched non-specific antibody. The vertical solid lines in (A) and (B) represent the mean fluorescence intensity of the MHC-I staining in Mock treated cells. These are representative data from one experiment repeated four times with similar results.



An active UPR decreases MHC class I cell surface expression

Thus far we had demonstrated that HFE C282Y induces a cellular UPR and that HFE C282Y expressing cells have reduced cell surface expression of MHC-I. To specifically test whether the MHC-I defect was a consequence of the UPR we applied two independent approaches. Firstly, we transfected cells to express active isoforms of the UPR signaling transcription factors ATF6 (nATF6) and XBP-1 (sXBP-1). Both proteins were independently capable of activating the UPR in transfected 293T cells as indicated by induced increases in BiP and GRP94 protein levels (Figure 6A, upper panel, second and third lanes respectively). Flow cytometry analysis revealed that both treatments were capable, along with C282Y expressing cells, of significantly ($P < 0.05$) decreasing MHC-I cell surface expression compared to mock treated cells (Figure 6B middle histogram and C). It is important to note that this effect was specific for cell surface MHC-I as the UPR induced by either misfolding C282Y or spliced XBP-1 did not appear to give rise to any corresponding changes in total MHC-I expression (Figure 6D).

While these combined results could point towards a general disruption of intracellular protein trafficking we found that TfR1 membrane expression was similar between UPR active and mock treated cells (Figure 6B lower histogram and C). This argues against a non-specific compromise in secretory protein pathways or general cell viability.

Blocking UPR signaling rescues surface MHC-I expression

These data supported our hypothesis linking non-specific UPR signaling with specific decreases in cell surface expression of MHC-I. Furthermore, having demonstrated in Figure 4 that C282Y promotes splicing of

XBP-1, and now showing in Figure 6 that spliced XBP-1 can decrease cell surface expression of HFE, we believed that we had experimental evidence specifically linking C282Y-associated UPR with reduced surface MHC-I. Nevertheless a second independent approach was applied to confirm this connection. Specifically we wondered whether blocking UPR signaling, through the use of a dominant negative XBP-1 protein, could rescue the decrease in surface MHC-I observed in C282Y expressing cells.

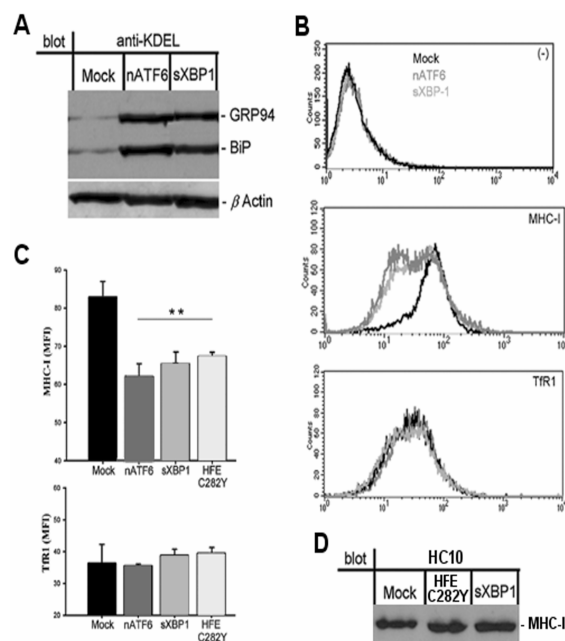


Figure 6 – UPR activation impairs the MHC-I cell surface expression (A) BiP and GRP94 from whole cell lysates of 293T cells transfected to express nATF6 and sXBP-1 were detected by Western blot with anti-KDEL. As a loading control, the membrane was striped and incubated with anti- β -Actin. The position of each protein is indicated on the right. A representative experiment of three made with similar results is shown. (B) 48 hours post-transfection with nATF6, sXBP-1 or the empty vector (Mock), 293T cells were surface stained and analyzed by flow cytometry. The upper histogram represents the negative control obtained by incubation with an isotype-matched non-specific antibody, the middle histogram represents the MHC-I cell surface expression and the lower histogram the Tfr1 staining. (C) The mean + 1SD of the MHC-I and Tfr1 mean fluorescence intensity (MFI) obtained in three independent experiments is shown. The asterisk represents a statistically significant difference between the MHC-I MFI obtained after each of the transfections and the mock cells ($P < 0.01$). (D) Total MHC-I from empty plasmid (Mock), HFE C282Y and sXBP-1 transfected 293T cells was detected by Western blot with HC10.

Figure 7 shows the results from transfection experiments performed to test this. In these experiments the decrease in surface MHC-I that was associated with C282Y expression was similar to the significant decreases achieved through treatment of mock transfected cells with DTT, or expression of the UPR signaling protein spliced XBP-1. Co-transfection of XBP-1-DN to block UPR signaling significantly rescued surface MHC-I expression in C282Y expressing cells. Similar results were obtained when a dominant negative ATF6 protein was co-transfected with HFE C282Y (data not shown).

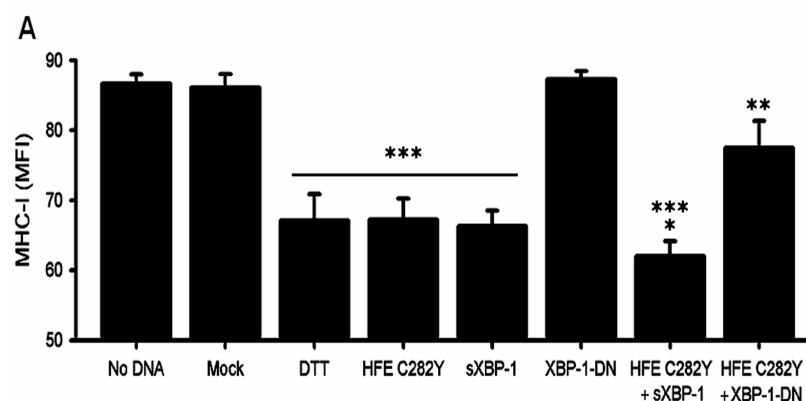


Figure 7 – UPR signaling blocking partially rescues cell surface MHC-I in HFE C282Y transfected cells 48 hours post-transfection with each indicated plasmid (No DNA refers to untransfected cells and Mock to cells transfected with the empty vector) or treatment with 2 mM DTT for 5 hours, 293T cells were surface stained with the mAb W6/32 and analyzed by flow cytometry. The mean + 1SD of the MHC-I mean fluorescence intensity (MHC-I MFI) obtained in three

independent experiments is shown. The asterisks represent statistically significant differences as follows: HFE C282Y vs HFE C282Y+XBP-1-DN, **, $P=0.0018$; Mock vs DTT/ HFE C282Y/ sXBP-1/ HFE C282Y+sXBP-1, ***, $P<0.001$. The value for HFE C282Y+sXBP-1 sample is significantly lower than HFE C282Y (*, $P<0.05$).

Discussion

Interest in the processes that regulate the protein folding, and in the cellular response that results when proteins misfold, is extending beyond basic tenets to application in understanding the molecular mechanisms of disease (Macario and Conway de Macario 2005). In this study a 293T cell line model system transiently expressing HFE wt or its C282Y mutant counterpart was used to analyze the cellular response to the stress imposed by misfolding of the HFE protein. The HFE C282Y mutant protein has previously been shown to be retained in the ER and middle Golgi compartment, and thus fails to proceed through the standard secretory pathway towards the cell membrane (Waheed et al. 1997). The statistically significant differences in BiP, GRP94 and CHOP expression levels that were observed between HFE wt and C282Y 293T expressing cells, and in PBMCs isolated from control (HFE wt) or HH patients (HFE C282Y homozygous) confirmed that an UPR is activated in response to the expression and accumulation of mutant HFE protein. BiP levels are normally under tight control at the level of protein translation. This homeostatic restriction is alleviated when the cell experiences ER stress (33). In these circumstances, enhanced translational efficiency results in higher protein production. This tight control of cellular BiP levels justifies the use of this protein as an UPR biological sensor and the magnitude and significance of the increases that we observed in HFE C282Y PBMCs provide strong evidence that the mutant protein is promoting ER stress conditions. We additionally noted that HFE C282Y expression resulted in the activation of the IRE1 signaling pathway as indicated by the alternative splicing of XBP-1.

The results obtained after introducing a MHC-I protein (HLA-A2) carrying a similar point mutation (C283Y) as HFE (C282Y), and hence prone to the same folding defect, further corroborates that this particular mutation is sufficient to abrogate the correct assembly and maturation of the protein, leading to the UPR activation. A link between the expression of a misfolded classical MHC-I protein and induction of the UPR was also recently demonstrated in the context of HLA-B27 (Turner et al. 2005).

With the purpose of investigating changes in MHC-I cell surface expression resulting from activation of the UPR, nATF6 or sXBP-1 were expressed on 293T cells. These experiments showed that UPR activation leads to the downregulation of membrane bound MHC-I molecules. This effect was specific for MHC-I cell surface expression as the UPR did not appear to affect total MHC-I expression. A comparable effect on MHC-I expression was observed in HFE C282Y transfected cells.

We have previously described that MHC-I cell surface expression is decreased in HFE C282Y mutant PBMCs (de Almeida et al. 2005). The impact of HFE wt and C282Y overexpression on MHC-I expression in 293T cells was assessed in the present study. The results showed a significant reduction in surface expressed MHC-I after the expression of each protein. The effect observed in HFE wt expressing cells is reverted by HFE wt/ β_2m co-transfection being attributed to the competition between HFE wt and MHC-I for binding with the available β_2m pool. A similar explanation for the MHC-I cell surface expression reduction in HFE wt overexpressing cells was

previously suggested (Vahdati-Ben Arieh et al. 2003). The MHC-I downregulation following HFE C282Y expression cannot be explained in the same way. Indeed, considering that the C282Y mutation impairs the HFE/ β 2m association, the overexpression of HFE C282Y protein should not lead to the depletion of the free β 2m pool. Instead, UPR activation appears to be the primary cause of decreased MHC-I in HFE C282Y expressing cells. The results obtained with the ATF6-DN (data not shown) and the XBP-1-DN expression vectors consolidate our conclusions that an UPR is the source of the MHC-I anomalies described in HFE C282Y mutant cells. Indeed, the strong correlation between the XBP-1 alternative splicing levels (Figure 4) and the reduction in MHC-I expression obtained in HFE C282Y or sXBP-1 expressing cells, or in DTT treated cells (Figure 7), suggests that UPR signaling pathways mediate this HFE C282Y/MHC-I cross-talk. Furthermore, the UPR induced by the HFE C282Y provides a corrective measure for an insult that occurred in the ER, while in the case of sXBP-1 or nATF6 expression the ER is not subjected to a stress of any kind. This indicates that the effect on MHC-I is not due to the stress conditions in the ER, but a direct consequence of the UPR signaling pathway. The exact mechanisms behind the UPR/MHC-I cross-talk warrants further investigation.

A relationship between the ER exit and the UPR, namely through the modulation of cargo proteins' levels, has already been described suggesting that this stress response affects protein traffic from and beyond the ER (Sato et al. 2002; Nyfeler et al. 2003). The finding that the time spent by MHC-I complexes in the ER determines the extent of the peptide loading optimization (Lewis and Elliott 1998) suggests that its reduced cell surface expression in HFE C282Y mutant cells is the outcome of an incomplete peptide optimization due to the premature ER release in UPR active cells. In fact, we have recently shown that, in HFE 282Y mutant cells, MHC-I peptide loading and ER egress occurs faster than in wt cells (24). Furthermore, thermostability assays and MHC-I endocytosis experiments showed that in the HFE mutant cells there is a premature MHC-I/peptide dissociation which is in accordance with the higher amount of free MHC-I heavy chains present at the cell surface and the increased MHC-I endocytosis rate observed in the HFE C282Y mutant cells (24).

The observed reduction of MHC class I expression as a consequence of the UPR may have some significance for work on viral immune evasion. In fact, several viruses lead to the activation of the UPR signaling pathway in infected cells. Although the reason for this is incompletely understood (Su et al. 2002; Tardif et al. 2002; Liu et al. 2004; Tirosh et al. 2005; Hegde et al. 2006), it may constitute one of the mechanisms that viruses have evolved to evade immune surveillance.

Several examples of UPR activation by mutations that compromise the correct folding and intracellular trafficking of the proteins have already been reported (Schroder and Kaufman 2005c). Most of them relate to a pathological condition either due to the protein's loss of function or through the implication in other cellular processes. Here we report that a point mutation (C282Y) in HFE leads to the UPR activation and that this mechanism has an impact on the cell surface expression of MHC-I molecules. Based on these findings, we suggest that the UPR activation that occurs in response to the HFE's misfolding of the C282Y mutant could be a contributing factor to the immunological defects reported in HH patients (Reimao et al. 1991; Arosa et al. 1994; Cardoso et al. 2001). A link between the UPR signaling pathway and MHC-I antigen presentation pathway is now established. The challenge for future studies is twofold: to elucidate the influence of the UPR on proteins involved

in iron metabolism and the oxidative stress associated with iron overload and to test the impact of the MHC-I defects on the reported anomalies in lymphocyte numbers linked to severe iron overload in HH.

CHAPTER 4

CHEMICAL CHAPERONES REDUCE ER STRESS AND PREVENT MUTANT HFE AGGREGATE FORMATION

de Almeida SF, Picarote G, Fleming JV, Carmo-Fonseca M, Azevedo JE, de Sousa M. *J Biol Chem*.

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Abstract

HFE C282Y, the mutant protein associated with Hereditary Hemochromatosis (HH), fails to acquire the correct conformation in the endoplasmic reticulum (ER) and is targeted for degradation. We have recently shown that an active unfolded protein response (UPR) is present in HH patients' cells. Now, using HEK 293T cells, we demonstrate that the stability of HFE C282Y is influenced by the UPR signaling pathway that promotes its degradation. Treatment of HFE C282Y expressing cells with tauroursodeoxycholic acid (TUDCA), a bile acid derivative with chaperone properties, or with the chemical chaperone sodium 4-phenylbutyrate (4PBA) impeded the UPR activation. However, while TUDCA led to an increased stability of the mutant protein, 4PBA contributed to a more efficient disposal of HFE C282Y to the degradation route. Fluorescence microscopy and biochemical analysis of the subcellular localization of HFE revealed that a major portion of the C282Y mutant protein forms intracellular aggregates. Although neither TUDCA nor 4PBA restored the correct folding and intracellular trafficking of HFE C282Y, 4PBA prevented its aggregation. These data suggest that TUDCA hampers the UPR activation by acting directly on its signal transduction pathway, while 4PBA suppresses ER stress by chemically enhancing the ER capacity to cope with the expression of misfolded HFE, facilitating its degradation. Together, these data shed light on the molecular mechanisms involved in HFE C282Y-related HH and open new perspectives on the use of orally active chemical chaperones as a therapeutic approach for HH.

Introduction

A large number of diseases result from protein misfolding and aggregation. Hereditary Hemochromatosis (HH) constitutes an example of such a disease. HFE, a type I transmembrane glycoprotein homologous to MHC-I, interacts with β_2 -microglobulin (β_2m) and only the HFE/ β_2m heterodimer is able to reach the cell surface through the standard secretory pathway (Feder et al. 1996). The C282Y mutation in HFE prevents the formation of an intra-molecular disulfide bridge in the $\alpha 3$ domain of HFE blocking β_2m association and the trafficking of the protein to the cell surface (Feder et al. 1997). The resulting misfolded protein is retained in the endoplasmic reticulum (ER) and subjected to accelerated proteasomal degradation (Waheed et al. 1997). The observation that HFE binds to transferrin receptor I (TfR1) implicated this protein in the regulation of iron metabolism (Feder et al. 1998), a fact supported by the finding that carriers of the HFE C282Y mutation develop HH, an iron overload disorder (Feder et al. 1996).

It has been known for some time that some compounds collectively called chemical chaperones have the ability to stabilize proteins in their native conformation contributing in some cases to rescue the folding defect of mutant proteins (Welch and Brown 1996). One well studied example of this mechanism is the restoration of the cell surface expression and function of the mutant cystic fibrosis transmembrane conductance regulator (CFTR) protein by chemical chaperones (Brown et al. 1996). It is thought, however, that these compounds may be effective in a number of other protein folding defects, thus providing an interesting therapeutic approach for a large number of different human diseases (Chaudhuri and Paul 2006).

We have recently reported that cells expressing HFE C282Y have an active unfolded protein response (UPR) (de Almeida et al. 2007). This specific ER stress response enhances the levels of molecular chaperones involved in protein folding and degradation and reduces the rate of protein synthesis (Schroder and Kaufman 2005b). Upon UPR activation, activating transcription factor-6 (ATF6), an ER-stress transducing protein, is cleaved (nATF6) and relocates to the nucleus where it promotes expression of UPR responsive genes (Haze et al. 1999; Shen et al. 2002). Another active transcription factor that promotes transcription of UPR responsive genes is produced by the alternative splicing of X box-binding protein-1 (sXBP1) (Yoshida et al. 2001).

Our aim in the present paper is to investigate the effect of the chemical chaperones tauroursodeoxycholic acid (TUDCA) and sodium 4-phenylbutyrate (4PBA) in the HFE C282Y-associated UPR as well as the impact of these compounds on the intracellular trafficking and localization of the HFE mutant protein. We show that chemical enhancement of the ER folding capacity results in the prevention of the UPR activation and influence the degradation of HFE C282Y. In addition, investigation of the sub-cellular localization of HFE C282Y revealed that this misfolded protein forms aggregates and that 4PBA is effective in preventing their formation. These findings offer a potential new strategy for therapy designed to prevent the potential toxicity of the intracellular aggregates.

Material and Methods

Antibodies and plasmids The following Abs were used: 8C-10 (Mouse anti-human HFE, a kind gift from Dr Rachel Ehrlich, Tel Aviv University, Israel); rabbit anti-HFE cytoplasmic tail (CT) (Ben-Arieh et al. 2001); mouse anti-KDEL (detects mainly BiP and GRP94) and rabbit anti- β -Actin (Abcam, Cambridge, UK); mouse anti-HA (Abcam, Cambridge, UK); donkey anti-mouse-FITC, anti-rabbit-FITC and donkey anti-mouse-Cy3 conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA);

The HFE wt-pcDNA3 construct was a kind gift from Dr Luísa Salter-Cid. HFE C282Y-pcDNA3 was described previously (de Almeida et al. 2007). β 2m-pcDNA3.1 constructs were a kind gift from Dr Hal Drakesmith (University of Oxford, UK). The pEP7-nATF6-FL vector expressing a nuclear targeted and transcriptionally active fragment of ATF6 (amino acids 1-373) was described previously (Fleming et al. 2004; de Almeida et al. 2007). A plasmid encoding the spliced form of XBP-1 was the kind gift of Dr K. Mori (Kyoto University, Japan) and acted as template for a *pfu* PCR amplification as described (de Almeida et al. 2007). The pEP7 HFE wt-GFP and pEP7 HFE C282Y-GFP vectors were generated by *pfu* PCR amplification using specific sense (GGTCAGATCTGGCCACCATGGGCCCGCGAGCCAGGCCG) and antisense (CCCCCTCGTCGACTCACGTTTCAGCTAAGACGTA) primers and the HFE wt-pcDNA3 and HFE C282Y-pcDNA3 constructs, respectively, as templates. Amplified products were cloned into the *Bgl*III and *Sall* sites of the pEP7-GFP vector. The pEP7-GFP vector was generated by *pfu* PCR amplification using the pEGFP-N1 (Clontech, Mountain View, CA) plasmid as template and the specific sense (GCGGGGTGACGATGGTGAGCAAGGGCGAGGAGCTG) and antisense (GGGCACCGCGGCCGCTTACTTGTACAGCTCGTCCATGCC) primers. Amplified GFP was cloned into the *Sall* and *Not*I sites of the pEP7-HA vector (Fleming and Wang 2003), thus replacing the carboxyl-terminus HA-tag with EGFP.

Cells Human Embryonic Kidney 293T cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM w/ GlutaMAX medium (Gibco, Grand Island, NY) containing 1% penicillin/streptomycin/amphotericin solution (Sigma-Aldrich, St. Louis, MO) and 10% heat-inactivated fetal bovine serum.

Transfections 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 60 mm plates accordingly to the manufacturer's protocol. At the time of transfection, cells were 90-95% confluent. Opti-MEM was used to dilute both DNA and Lipofectamine at a final DNA:Lipofectamine ratio of 1:2.5. After transfection cells were incubated for 48 hours in DMEM w/ GlutaMAX medium (Gibco, Grand Island, NY) containing 1% penicillin/streptomycin/amphotericin (PSA) solution (Sigma-Aldrich, St. Louis, MO) and 10% heat-inactivated fetal bovine serum (FBS). In these studies a plasmid encoding β 2m was co-transfected with HFE expression vectors in order to ensure that sufficient quantity of this protein was available for HFE's correct assembly.

Pulse Chase 48 hours after transfection, 1×10^7 293T cells were starved for 1 hour in Cysteine/Methionine free DMEM medium (Gibco, Grand Island, NY) supplemented with 1% L-glutamine and pulsed for 20 minutes with 140 $\mu\text{Ci/ml}$ Pro-Mix L- ^{35}S Cysteine/Methionine (Amersham Biosciences UK, England). The culture medium was then supplemented with cold cysteine and methionine and chased for the indicated times. At each time point one aliquot was taken, washed and lysed in ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Triton-X, Complete EDTA Free-protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 10 mM iodoacetamide). Cell debris were removed by centrifugation and the lysates pre-cleared with 100 μl protein-A Sepharose beads slurry (50%) for 1 hour at 4°C. The lysates were incubated overnight at 4°C with anti-HA. Immunocomplexes were pulled down with protein-A sepharose beads and washed three times in ice-cold lysis buffer. After addition of gel loading buffer solution (Laemmli 1970) with 10% β -mercaptoethanol and boiling for 5 minutes, samples were loaded on 10% SDS-PAGE.

SDS-PAGE and Quantitation 10% SDS-PAGE was performed using a Bio-Rad Mini Protean II kit. Gels were fixed in 10% acetic acid, 40% methanol, incubated for 30 minutes with Amplify solution (Amersham Biosciences UK, England) dried and exposed to a radioactivity storage screen. Quantitation was performed using a Typhoon phosphoimager (Molecular Dynamics, Sunnyvale, CA) with ImageQuant v5.1 software.

Western Blot, Endo H digestion and Ultracentrifugation Protein concentration in whole cell lysates was determined with RC/DC Protein Assay (Bio-Rad, Hercules, CA) and 30 μg separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose Hybond-C membrane (Amersham Biosciences UK, England). After blocking at 4°C with 5% dry milk/0.05% Tween 20 in TBS (TBS-T), the membrane was incubated with anti-HA or anti-KDEL, washed 3 times with TBS-T and detected with the respective HRP conjugated secondary antibody (Molecular Probes, Eugene, OR) and an enhanced chemiluminescence substrate (Pierce, Rockford, IL). To control loadings, the membrane was stripped using Restore WB Stripping Buffer (Pierce, Rockford, IL) and incubated with anti- β -Actin. For the Endo H assay whole cell lysates were digested for 4 hours at 37°C with Endo H (Roche Diagnostics, Mannheim, Germany) or Glycosidase F (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer. The reaction was stopped by addition of gel loading buffer solution (GLB) (Laemmli 1970) with 10% β -mercaptoethanol and boiling for 5 minutes.

For detection of protein aggregates, cells were solubilized in 1% Triton-X lysis buffer for 1 hour on ice. After a centrifugation step at 300g for 5 minutes to clear cell debris, whole cell lysates were split into two equal portions. One represented the total protein content and the other was ultracentrifuged at 100000g for 1 hour at 4°C in a Sorvall Ultra Pro80 centrifuge. The pellet fraction and the protein content of the supernatant fraction (obtained by a 10% trichloroacetic acid precipitation) were solubilized with GLB, 10% β -mercaptoethanol and boiling for 5 minutes. Equivalent gel loading was confirmed by staining the nitrocellulose membrane with 0,1% Ponceau S.

Treatment with chemical chaperones 18 hours post-transfection, 293T cells were washed and incubated for 30 hours in DMEM w/ GlutaMAX medium supplemented with 1% PSA and 10% FBS in the presence of 1mM TUDCA (Calbiochem, San Diego, CA) or 5mM 4PBA (Sigma-Aldrich, St. Louis, MO). The percentage of transfected cells was not affected by the treatment with the chemical chaperones (data not shown).

Real-time PCR Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Following treatment with 2U/sample of RQ1 DNase, in the presence of 50 U/sample of RNase inhibitor (Invitrogen), for 30 min at 37°C, 1 µg of RNA was reverse-transcribed, using Superscript reverse transcriptase (Invitrogen), following the manufacturer's instructions. Expression levels were evaluated by quantitative real-time PCR with the ABI PRISM 7700 instrument (Applied Biosystems, Forster City, CA, USA) using 1x SYBR Green PCR Master Mix (Applied Biosystems). Quantification of β -Actin gene expression was performed as a control. Relative expression levels were calculated as $2^{-(Ct \text{ human } \beta\text{-Actin} - Ct \text{ HLA-A} * 10000)}$ (for details see ABI PRISM 7700 - User Bulletin #2). The oligonucleotides used were 5'-CTCCTTTGGTGAAGGTGACACATC -3' and 5'-ATCACAAATGAGGGGCTGATCC-3' for HFE and 5'-CCTGGGTGGCGGAACCTTCGATGTG-3' and 5'-CTGGACGGGCTTCATAGTAGACCGG-3' for BiP.

Flow cytometry 293T cells transiently expressing GFP-tagged HFE wt or C282Y were washed in ice cold PBS, 0.2% BSA, 0.1% NaN₃ followed by incubation at 4°C with a saturating amount of primary Ab for 30 minutes in 96-well plates. After 3 washes cells were incubated with Cy3-conjugated secondary Ab for 30 minutes on ice without permeabilization. Cells were washed and flow cytometry analysis performed in a FACScalibur (Becton Dickinson, Mountain View, CA). The Cy3 fluorescence, representing cell surface expression of HFE, was measured in GFP positive cells. For each sample a minimum of 15 000 events were acquired. To define the background staining, irrelevant mAbs of the same isotype were used.

Fluorescence microscopy HFE wt-GFP or HFE C282Y-GFP transiently transfected 293T cells were grown on coverslips and either left untreated or treated with 1mM TUDCA or 5mM 4PBA for 30 hours. Cells were rinsed 3 times in PBS, fixed in 3,5% paraformaldehyde, and simultaneously blocked and permeabilized with 5% BSA, 0.1% Triton X-100 in PBS, for 30 min at RT. Fixed cells were incubated for 30 min with anti-KDEL, washed twice with PBS and incubated with anti-Mouse-Cy3 conjugated Ab. Coverslips were mounted on glass slides in 1:5 DAPI (10 µg/ml; Sigma-Aldrich):Vectashield (Vector Laboratories, Burlingame, CA) and immunofluorescence analysis performed on a Axiovert Carl Zeiss microscope (Carl Zeiss, Thornwood, NY). For quantification of cells containing HFE aggregates, at least 200 cells were counted according to an unbiased systematic random sampling scheme.

Statistical analysis To test the significance of the differences observed the Student's T-test was used. In all tests the statistical significance was 2-sided and considered at $P < 0.05$. Data are displayed as mean $\pm$ 1SD.

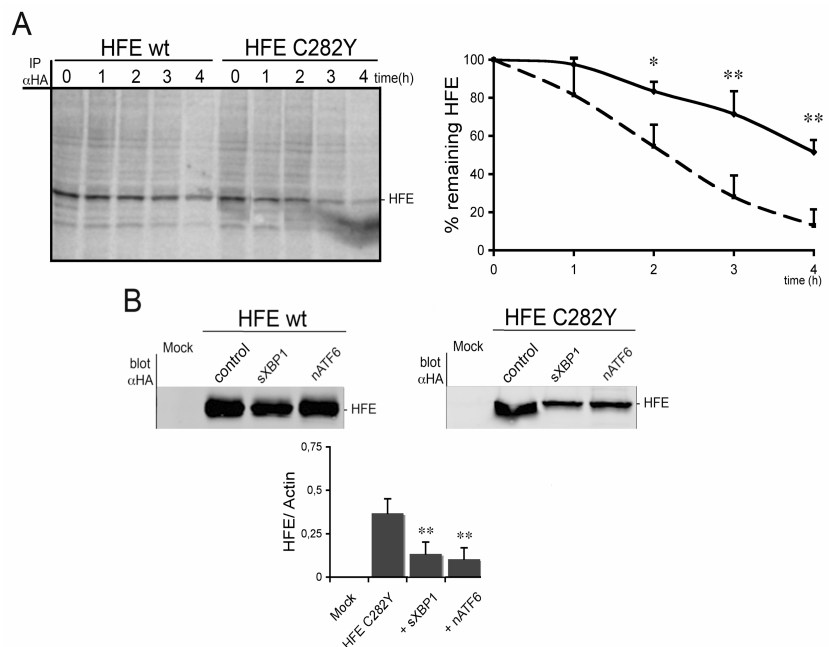
Results

Modulation of the UPR interferes with HFE C282Y stability

Previous studies have shown that HFE C282Y fails to travel beyond the ER and is subjected to accelerated proteasomal degradation in COS7 cells (Waheed et al. 1997). Pulse chase analysis performed in 293T cells confirmed these observations specifically in our transfected cell model system (Figure 1A). In 293T cells overexpressing HFE wt or C282Y, only the wt protein can reach the cell surface as observed by flow cytometry (Supplementary figure 1A) and by the HFE wt resistance to Endo H digestion (Supplementary figure 1B). In contrast, no Endo H resistant protein is observed in HFE C282Y transfected cells, confirming that the mutant protein is unable to leave the ER through the standard secretory pathway (Supplementary figure 1B).

Figure 1 – UPR activation promotes HFE C282Y degradation

(A) HFE wt and HFE C282Y transfected cells were ^{35}S labeled and chased for the indicated times. HFE was recovered by immunoprecipitation with anti-HA mAb. The graph represents the percentage of remaining protein at each time point (solid line – HFE wt; dashed line – HFE C282Y). Data are from 3 independent experiments. (* $p < 0.05$; ** $p < 0.01$). **(B)** 293T cells were transfected to express either HFE wt or HFE C282Y plus the plasmids indicated. The left and right hand side blots are derived from the same experiment (representative of 3 independent experiments). The blots were stripped and incubated with an antibody against β -Actin and the intensity of the HFE C282Y



bands was normalized to β -Actin levels and plotted on a bar graph as average + 1SD of three independent experiments. The asterisks represent statistically significant differences (** $p < 0.01$) compared to HFE C282Y transfected cells.

We have recently shown that the ER retained HFE C282Y mutant protein leads to the activation of the UPR (de Almeida et al. 2007). To investigate the involvement of the ER stress pathway on the HFE protein's stability, 293T cells were co-transfected with plasmids encoding HFE wt or C282Y and the UPR was genetically modulated by the co-expression of transcriptionally active isoforms of either ATF6 (nATF6) or XBP1 (sXBP1). Induction of the UPR was confirmed by analysis of GRP94 and BiP levels by western blotting (Supplementary figure 1C). Stimulation of the UPR by either sXBP1 or nATF6 resulted in highly significant decreases in steady state expression of the HFE C282Y protein compared to HFE wt (Figure 1B).

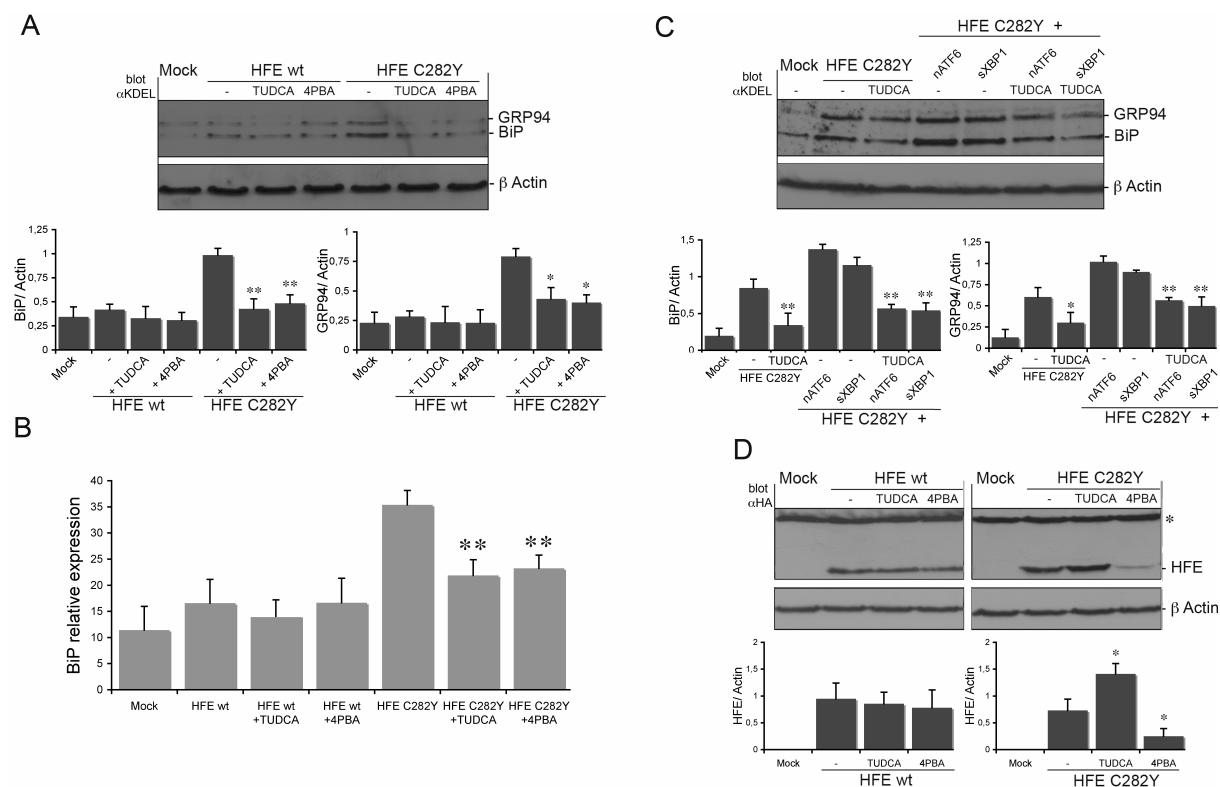


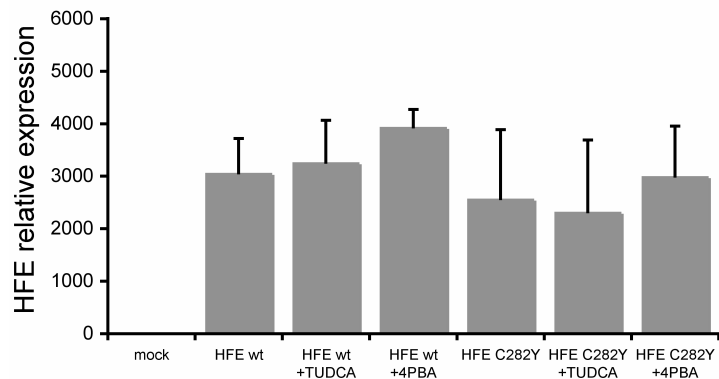
Figure 2 – Chemical chaperones protect from UPR activation and modulate HFE C282Y protein levels (A) BiP and GRP94 from whole cell lysates of HFE wt or C282Y transfected cells either untreated or treated with 1mM TUDCA or 5mM 4PBA were detected by western blot with anti-KDEL. The intensity of the bands was quantified, normalized to β -Actin, and plotted as average + 1SD. Data are from three independent experiments. The asterisks represent statistically significant differences (* p <0.05; ** p <0.01) between the cells treated with the chemical chaperones and the untreated cells. (B) BiP mRNA levels of 293T cells transfected with empty vector (Mock), HFE wt or HFE C282Y either left untreated or treated with the chemical chaperones TUDCA and 4PBA, were assessed by quantitative real-time PCR. The graph represents the average + 1SD of three independent experiments. β -Actin was used as an endogenous control gene. ** p <0.01 relatively to HFE C282Y untreated cells. (C) Cells transfected to express the indicated proteins were either treated with 1mM TUDCA or left untreated and BiP and GRP94 levels detected by western blot. The intensity of the bands corresponding to BiP and GRP94 was quantified, normalized to β -Actin and plotted on a bar graph as average + 1SD of three independent experiments. * p <0.05 and ** p <0.01 relatively to untreated cells expressing the same proteins. (D) HFE protein levels of HFE wt or C282Y transfected cells either untreated or treated with TUDCA or 4PBA were detected by western blot with anti-HA mAb. The asterisk indicates the position of a non-specific band. HFE band's intensity was normalized to β -Actin and plotted on a bar graph as average + 1SD of three independent experiments. * p <0.05 compared to HFE C282Y transfected cells.

Chemical chaperones block the UPR activation and alter the HFE C282Y stability

To evaluate the effect of chemical chaperones on the ER stress response, HFE wt or C282Y expressing cells were cultured in the presence of TUDCA and 4PBA. Using BiP and GRP94 levels as markers for the UPR activation, we observed that HFE C282Y transfected cells treated with chemical chaperones have lower levels of both proteins indicating that these compounds were effective in inhibiting the activation of the UPR in HFE C282Y expressing cells (Figure 2A). As an independent measure of UPR activation, real-time PCR was performed to quantify BiP expression at the transcriptional level. While no significant effect was observed in HFE wt transfected cells, analysis of the data showed a statistically significant decrease in BiP mRNA in HFE C282Y transfected cells cultured in the presence of TUDCA or 4PBA when compared to untreated cells (Figure 2B). The efficiency of chemical chaperones was further confirmed by the capacity of TUDCA to alleviate ER stress in cells co-expressing HFE C282Y plus nATF6 or sXBP1 (Figure 2C).

Having shown in figure 1 that stimulation of the UPR lowers the levels of HFE C282Y, treatment with chemical chaperones should rescue the mutant protein from ERAD. Indeed, we observed increased levels of this protein in HFE C282Y transfected cells when TUDCA was present in the culture medium (Figure 2D, right hand side blot, lane 2 vs. lane 3). However, treatment with 4PBA did not result in the stabilization of the HFE C282Y protein. In the presence of this chemical chaperone the amount of protein detected was smaller than that observed in untreated cells (Figure 2D, right hand side blot, lane 2 vs. lane 4). HFE wt transfection did not result in the UPR activation (Figure 2A). Accordingly, neither TUDCA nor 4PBA had any detectable effect on the protein levels of BiP, GRP94 (Figure 2A) and HFE wt (Figure 2D, left hand side blot).

Figure 3 - Chemical chaperones do not affect the transcription efficiency of HFE. 293T cells transfected with empty vector (Mock), HFE wt or HFE C282Y were either left untreated or incubated for 30 hours in the presence of 1 mM TUDCA or 5mM 4PBA, and HFE mRNA levels were assessed by quantitative real-time PCR. The graph represents the average + 1SD of three independent experiments. No statistically significant differences were observed. β -Actin was used as an endogenous control gene.



In order to exclude a possible effect of the chemical chaperones on the transcription efficiency of *HFE*, mRNA from TUDCA or 4PBA-treated and -untreated cells was quantified by real-time PCR. Figure 3 shows that no significant differences were observed in the HFE mRNA levels, corroborating the hypothesis that 4PBA facilitates HFE C282Y degradation.

Chemical chaperones do not restore HFE C282Y cell surface expression

Several examples of mutant proteins whose folding defects are corrected by the action of chemical chaperones have already been described (Welch and Brown 1996). As the C282Y mutation impairs the correct assembly of HFE molecules and concomitantly their trafficking beyond the ER towards the cell surface, we wanted to investigate if treatment with TUDCA or 4PBA promoted the stabilization of a conformation that restores HFE C282Y cell surface expression. To do that, 293T cells transiently expressing HFE wt-GFP or HFE C282Y-GFP were cultured in the presence or absence of TUDCA or 4PBA. Cell surface expression of HFE was then evaluated by flow cytometry analysis of the anti-HFE staining in GFP-positive cells (cells successfully transfected with HFE wt or HFE C282Y). The results revealed that treatment with the chemical chaperones had no effect on the correct cell surface expression of HFE wt when compared to untreated cells (Figure 4A). Regarding the effect of TUDCA and 4PBA on HFE C282Y, this set of experiments revealed that none of the chemical chaperones used was able to restore the cell surface expression of the mutant protein (Figure 4A). The failure of these compounds to promote the correct intracellular trafficking of HFE C282Y was further confirmed by the results obtained with Endo H. Following digestion with this glycosidase, analysis of protein extracts of cells expressing

HFE C282Y and cultured in the presence or absence of TUDCA or 4PBA did not reveal any Endo H-resistant HFE protein, as observed by the conversion of all the protein to a deglycosylated (HFE-CHO) state with higher electrophoretic mobility (Figure 4B, lane 1 vs lanes 2-4). These data strongly suggest that chemical chaperones cannot overcome HFE C282Y ER retention.

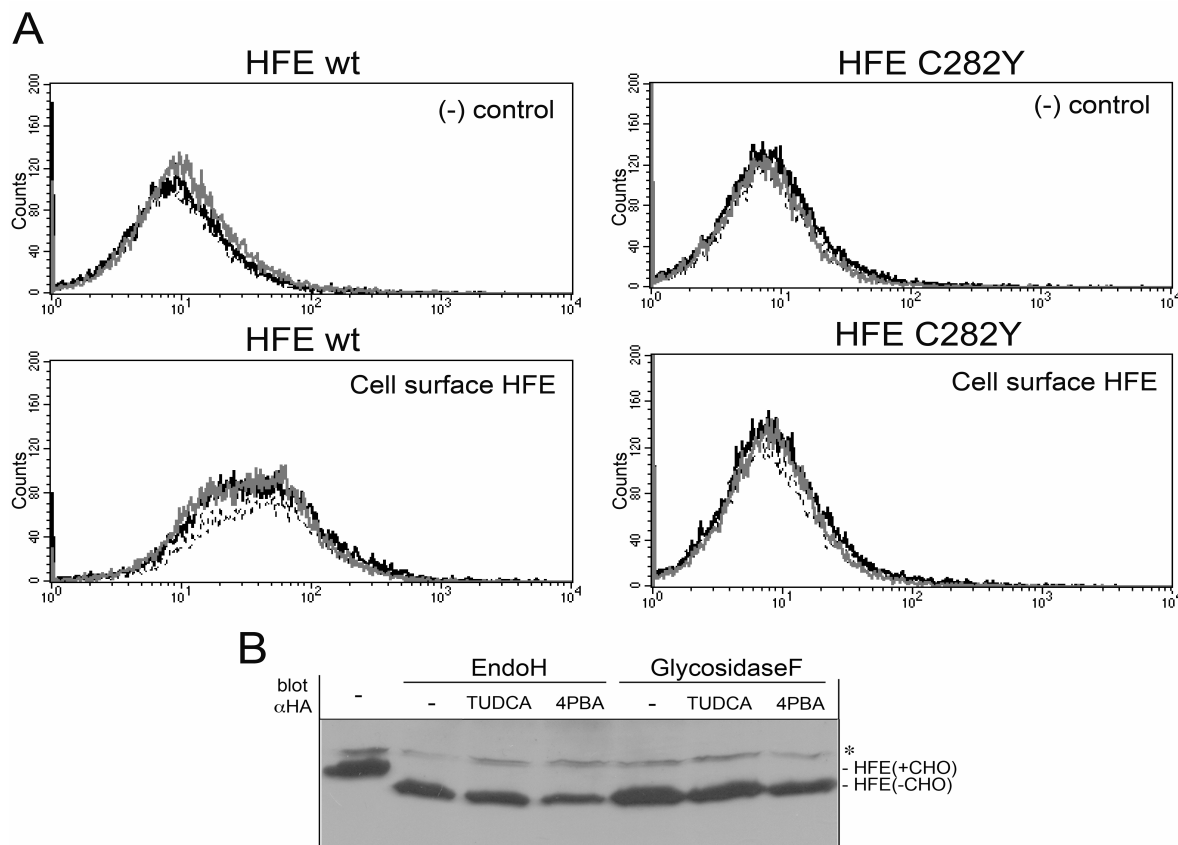


Figure 4 – Chemical chaperones do not restore the cell surface expression of HFE C282Y (A) 293T cells transfected with HFE wt-GFP or HFE C282Y-GFP were either left untreated or treated with chemical chaperones and stained without permeabilization using anti-HFE Abs plus Cy3-conjugated secondary Abs. GFP positive cells were gated and the HFE cell surface expression analyzed by flow cytometry. The negative (-) control was obtained by incubation with an isotype-matched non-specific antibody. The black lines in each histogram represent the untreated cells, grey lines represent TUDCA treated cells and dashed lines represent 4PBA treated cells. These are representative data from three independent experiments performed. **(B)** Whole cell lysates of 293T cells transfected with HFE C282Y and either left untreated or treated with chemical chaperones were digested with Endo H or Glycosidase F. Western blot was then performed using anti-HA mAb. The position of the Endo H-resistant (+CHO) and –sensitive (-CHO) forms of HFE is indicated. The asterisk indicates the position of a non-specific band. One representative experiment of three performed with similar results is shown.

4PBA prevents HFE C282Y aggregation

To investigate the intracellular localization of the HFE C282Y protein, 293T cells expressing either HFE wt-GFP or HFE C282Y-GFP were stained with anti-KDEL Ab and analyzed by immunofluorescence microscopy. KDEL is an ER retention motif found in proteins such as BiP and GRP94. The amount of GFP-tagged HFE proteins that co-localize with KDEL-containing proteins represents the portion of HFE residing in the ER. As expected, HFE wt showed only mild localization in the ER, with most of the protein spread out within the cell (Figure 5A, HFE wt). Contrary to this expression pattern, HFE C282Y was found in the ER (Figure 5A, HFE C282Y). Interestingly, however, we observed that in approximately 37% of the cells (Figure 5B), HFE C282Y was

found in intracellular aggregates (Figure 5A, arrows in the HFE C282Y panels) that co-localized extensively with KDEL proteins. Ultracentrifugation analysis of detergent treated protein extracts confirmed that this structures were the result of HFE C282Y aggregation (Figure 6). Indeed, the majority of the mutant protein was recovered in the pellet (detergent-resistant) fraction, contrasting with the higher amount of HFE wt observed in the supernatant (Figure 6A, lanes 5 vs 8 and 6 vs 9).

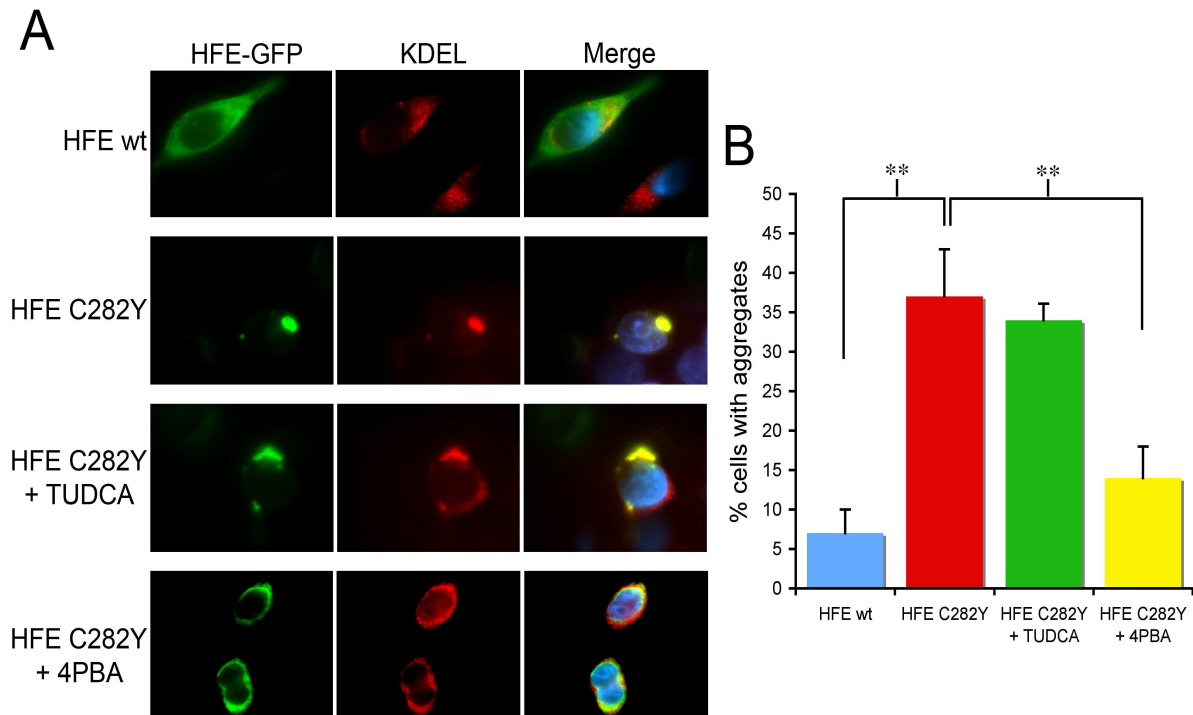


Figure 5 – 4PBA prevents the formation of HFE C282Y intracellular aggregates. 293T cells were transiently transfected with HFE wt-GFP or HFE C282Y-GFP and either left untreated or incubated for 30 hours in the presence of 1mM TUDCA or 5mM 4PBA. Permeabilized cells were stained with anti-KDEL (Red). Merged images are shown in yellow. DAPI staining of the cell nucleus is shown in blue. **(B)** Cells bearing intracellular aggregates were counted and percentages calculated. At least 200 cells from each experimental condition were counted. Cells showing accumulation of the HFE-GFP fluorescence in individual locus were classified as having protein aggregates. Data are from three independent experiments. (** $p < 0.01$).

Treatment with TUDCA did not produce any considerable change in the localization pattern or in the percentage of HFE C282Y aggregates (Figure 5A, HFE C282Y+TUDCA; Figure 5B). However, while no effect on the intracellular location of HFE C282Y was found in cells treated with 4PBA, this chemical chaperone significantly reduced the percentage of cells with detectable protein aggregates (Figure 5A, HFE C282Y+4PBA; Figure 5B), suggesting that 4PBA may promote a more efficient disposal of HFE C282Y to the degradation route, preventing its aggregation.

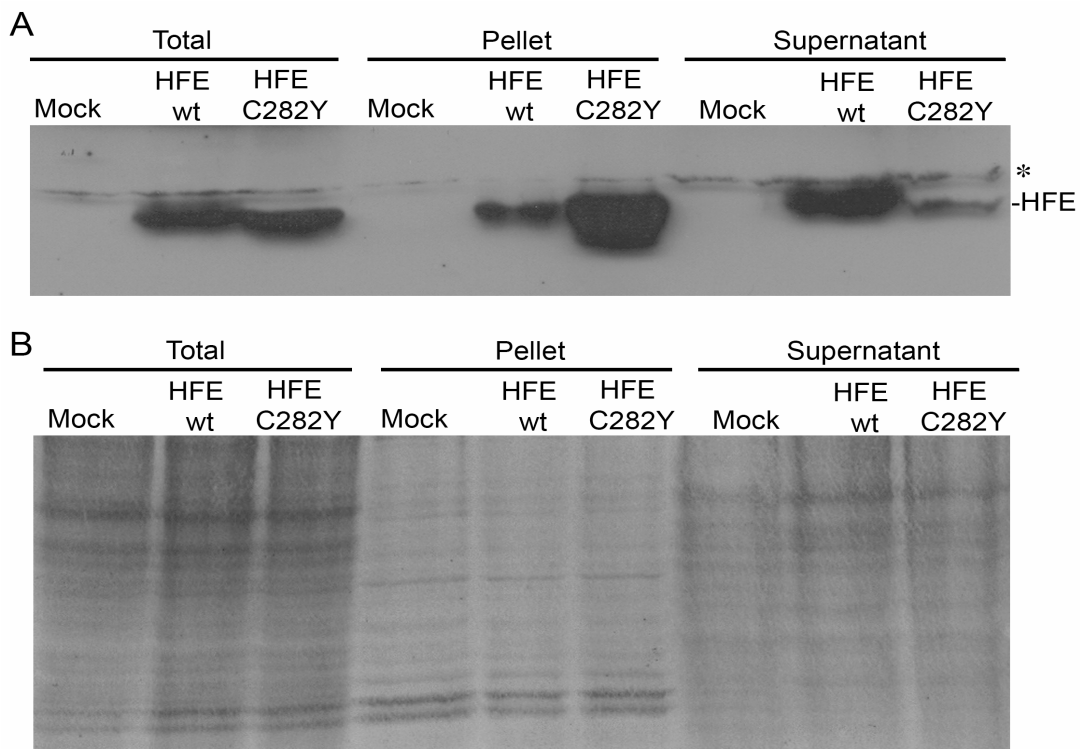


Figure 6 – Biochemical analysis of the HFE C282Y aggregates (A) Total cell lysates of HFE wt or C282Y transfected cells were solubilized with lysis buffer containing 1% Triton-X and centrifuged at 100000g for 1 hour at 4°C. HFE protein from total cell lysates (not centrifuged - Total), pellet fraction of the ultracentrifugation (Pellet) and the supernatant fraction (Supernatant) was detected by western blot with anti-HA mAb. The position of the HFE band is indicated. The asterisk indicates the position of a non-specific band. **(B)** As a loading control, the membrane was stained with 0.1% Ponceau S before blocking. Three individual experiments were performed with similar results.

Discussion

We have recently shown that an UPR is triggered following HFE C282Y expression (de Almeida et al. 2007). With this study we aimed at investigating the impact of this ER stress response, stimulated by genetic manipulation of 293T cells, on the fate of the mutant protein. In addition, the effects of two different chemical chaperones, TUDCA and 4PBA, on the HFE C282Y-associated UPR and intracellular distribution were also analyzed.

Herein we describe that in HFE C282Y expressing cells, activation of the ER stress response stimulates the clearance of the mutant protein. Confirming the direct link between the UPR and the HFE C282Y degradation, we observed that this effect is amplified in the presence of nATF6, sXBP1.

Here we report that HFE C282Y forms intracellular aggregates in vitro. The co-localization pattern obtained for HFE C282Y and KDEL-containing proteins suggests that the protein aggregates contained, besides HFE C282Y, ER resident proteins. Since we found anti-KDEL to detect BiP to a greater extent than other KDEL-containing proteins by western blot and native immunoprecipitation experiments (de Almeida et al. 2007), it is reasonable to assume that BiP is one of the major components of the observed aggregates. In fact, it was previously observed that protein aggregates are enriched in molecular chaperones (Garcia-Mata et al. 1999). A common feature of almost all diseases of protein conformation is the formation of aggregates caused by destabilization of the α -helical structure with simultaneous formation of a β -sheet (Dobson 1999). These aggregates tend to resist degradation and accumulate in inclusion bodies, which are usually present in low copy number, most often, only one per cell (Kopito 2000).

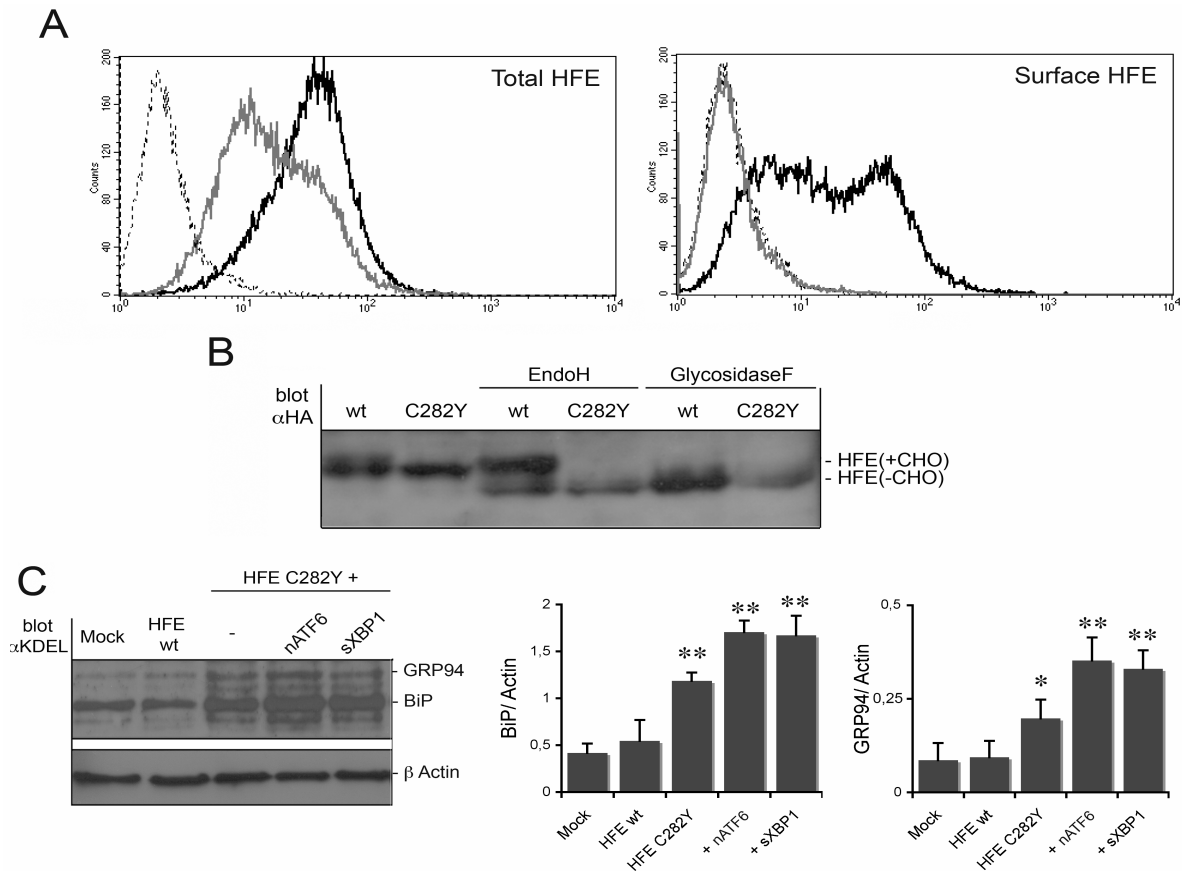
Chemical chaperones, such as 4PBA, are a group of compounds known to improve ER folding capacity and facilitate the trafficking of mutant proteins by stabilizing their conformation (Welch and Brown 1996). 4PBA was previously shown to increase the trafficking of a mutant cystic fibrosis transmembrane regulator (CFTR Δ 508) (Rubenstein et al. 1997) and to enhance the secretion of the mutant α 1-ATZ protein (Burrows et al. 2000). Endogenous bile acids derivatives, such as TUDCA, can also modulate ER function protecting from UPR induction and ER stress-induced apoptosis (Xie et al. 2002; Ozcan et al. 2006). In this study, we investigated whether pharmacologically active chemical chaperones could alleviate the ER stress response and their effect on the fate of the HFE C282Y protein. We show that both TUDCA and 4PBA protect from UPR activation in HFE C282Y expressing cells. In addition, we show that the stability of the HFE mutant protein is modulated by this small molecular weight compounds. Chemically improving the ER folding capacity did not result in the restoration of HFE C282Y correct intracellular trafficking and cell surface expression. However, an effect was observed on the efficiency of disposal of the mutant protein to degradation. While TUDCA leads to an increase in the protein's levels, 4PBA seem to act by facilitating the degradation of the misfolded HFE. 4PBA prevented the formation of intracellular protein aggregates, possibly accounting for a more competent targeting of HFE C282Y to the degradation route. Protein misfolding and aggregation is known to be associated with several other diseases as is the case of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) or Huntington's disease (HD) (Glabe and Kaye 2006). Although the identity of the aggregates and the mechanism

by which they disable and eventually kill a neuron are unknown, compiling evidence strongly suggests that these aggregates may be toxic (Caughey and Lansbury 2003). Heat shock proteins (HSPs) represent one class of proteins whose expression is upregulated in response to several types of stress and that are involved in the cellular quality control machinery that shepherds protein folding, avoids aggregation or targets misfolded proteins for degradation (Morimoto and Santoro 1998; Bukau et al. 2006). It was reported previously that one member of this class of proteins, the 70-kDa heat shock protein (HSP70), has a protective role against polyglutamine protein aggregation and toxicity in HD models (Sakahira et al. 2002). The ability of HSP70 to prevent aggregation may be of particular significance here, since 4PBA treatment was previously shown to induce total cellular 70-kDa heat shock protein (HSP70) expression (Choo-Kang and Zeitlin 2001). Furthermore, as mentioned before, although the mechanism of action of chemical chaperones remains largely unknown, 4PBA has been shown to prevent aggregation of denatured alpha-lactalbumin and bovine serum albumin (Kubota et al. 2006). Together with the results reported here, namely the decrease in the HFE C282Y protein levels and intracellular aggregates, these findings support the assumption that 4PBA protects from HFE C282Y-induced UPR by increasing the efficiency of the quality control machinery that ultimately leads the mutant protein to the degradation route thus removing the ER stress stimulus.

Previous studies have demonstrated that TUDCA has the capacity to protect from ER stress (Xie et al. 2002; Ozcan et al. 2006). However, unlike 4PBA, TUDCA has not been described before to act as a chaperone that promotes the folding and trafficking of mutant proteins. Likewise, in the present study we did not observe any significant effect of TUDCA on HFE C282Y's intracellular trafficking or localization. Instead, TUDCA was found to increase the mutant protein's stability possibly as a consequence of its influence on the UPR activation that, as shown here, enhances the clearance of the mutant HFE. Whilst with a similar outcome, the mechanism of action of TUDCA and 4PBA leading to the UPR suppression seem to diverge. TUDCA appears to prevent the UPR activation by acting directly on its signal transduction pathway. 4PBA accomplishes the same goal by modulating the ER capacity to cope with the expression of misfolded HFE, facilitating its degradation.

Despite the failure of the chemical chaperones to restore the cell surface expression of HFE C282Y, both TUDCA and 4PBA were effective in decreasing the magnitude of the HFE C282Y-associated UPR. In addition, 4PBA treatment prevented the formation of putatively toxic intracellular aggregates. However, the connection between the UPR activation and HH is recent (de Almeida et al. 2007). The clarification of the physiological significance and contribution of both the UPR and the mutant HFE aggregates to the pathophysiology of HH will certainly clarify the therapeutic potential of these chemical chaperones.

Supplementary data



Supplementary Figure 1 – HFE C282Y is retained in the ER and subjected to accelerated degradation (A) HFE wt and HFE C282Y transfected cells were stained with anti-HFE Abs with (total HFE) or without (surface HFE) permeabilization (with 0.2% Saponin) and analyzed by flow cytometry. The black lines in each histogram represent the HFE wt expressing cells and the grey lines represent the HFE C282Y expressing cells. The negative (-) control is represented in the histograms by a dashed line. One representative experiment of three performed with similar results is shown. (B) Whole cell lysates of 293T cells transfected with HFE wt or HFE C282Y were digested with Endo H or Glycosidase F. The protein was visualized by western blot using an anti-HA mAb. The positions of the Endo H-resistant (+CHO) and -sensitive (-CHO) forms of HFE are indicated. Three independent experiments were performed with similar results. Glycosidase F digestion allows the identification of the fully deglycosylated form of HFE in the blot. (C) To evaluate the UPR activation in 293T cells transfected with the indicated plasmids, the levels of BiP and GRP94 were assessed by western blot with anti-KDEL mAb. The position of both proteins in the gel is indicated. The intensities of the bands corresponding to BiP and GRP94 were normalized to β -Actin levels and plotted on a bar graph as average + 1SD of three independent experiments. The asterisks represent statistically significant differences (* p <0.05; ** p <0.01) of BiP and GRP94 increase compared to mock cells.

CHAPTER 5

GENERAL DISCUSSION

First identified in the context of HH, HFE quickly became captive of its own discovery. Notwithstanding the growing amount of evidence favoring the involvement of this non-classical MHC-I protein in other unrelated processes, the link to HH and more significantly its role as a modulator of transferrin-bound iron uptake have established HFE as an *iron protein*. But nature has limited resources to successfully design a rather complex enterprise: the living organism. Multitasking seems inevitable if the enormous number of biological processes is to be accomplished by the relatively scarce amount of proteins/genes. Similarly to the “*one gene - one protein*” binomial, the idea of a “*one protein - one function*” is rapidly falling apart by the identification of several proteins involved in more than a single physiological event.

The amino-acid sequence identity and structural homology to MHC-I, the immunological abnormalities observed in HH patients and the close relationship between viruses and HFE have propelled the hypothesis that its function might go beyond the exclusive participation in iron homeostasis. To evaluate the putative involvement of HFE on immunological processes specifically by investigating the influence of the C282Y mutation on selected events was the original aim of the present thesis. The results obtained and their physiological significance will now be discussed.

Mounting evidence suggests the involvement of HFE in immunological processes

Alongside the well-characterized deficiency to successfully utilize iron, several immunological defects have been previously found in HH patients. Altered CD4+/CD8+ ratios (Reimao et al. 1991), diminished CD8+ T-lymphocytes' cytotoxic activity (Arosa et al. 1997), decreased CD8-p56lck activity (Arosa et al. 1994), and altered TCR repertoire in C282Y carriers (Cardoso et al. 2001) are distinctive features of HH, even though their molecular basis is not known. One characteristic of these defects is the involvement of an individual subset of lymphocytes: the CD8+ T-lymphocytes. A proficient interplay between CD8+ T-lymphocytes and MHC-I is vital for the success of the immune response against invading pathogens. Moreover, the TCR and the p56lck (determinant for the cytotoxic response following MHC-I/peptide recognition) are specifically distressed by the faulty molecular mechanism present in HH patients. Altogether, this collection of evidence pointed to the existence of MHC-I abnormalities that could contribute to the clarification of the immunological defects associated with HH. In fact, a thorough analysis of the MHC-I antigen presentation pathway revealed the existence of significant differences between HFE C282Y mutant cells obtained from HH patients and HFE wt cells. A myriad of corrupted intracellular events leads to the generation of high off-rate MHC-I/peptide complexes that dissociate prematurely during their journey towards the cell surface. The outcome is a diminished surface expression of MHC-I molecules in HFE C282Y mutant cells (Chapter 2). Although not yet tested, the key role exerted by MHC-I molecules in a number of processes suggests that this defect may have a considerable physiological significance. In view of that, a correlation between this observation and the recognized immunological defects linked to HH may be discussed.

Signaling by MHC-I/peptide complexes determines the nature of the CD8+ T-lymphocytes immune response. It is reasonable to propose that impaired expression of these complexes in the context of HH may

constitute the molecular basis for the diminished CD8⁺ T-lymphocytes' cytotoxic activity observed in HH patients (Arosa et al. 1997). Likewise, this would lead to decreased CD8-p56lck activity, which is also acknowledged as a feature of HH (Arosa et al. 1994). The importance and physiological significance of the interaction between the TCR in CD8⁺ T-lymphocytes and MHC-I molecules extends beyond the mere immune surveillance mechanism, playing a decisive role in processes such as the maintenance of the CD8⁺ T-lymphocytes pool, the shaping of the TCR repertoire or the natural killer (NK) cells activity (Akolkar et al. 1993; Gulwani-Akolkar et al. 1995; Tanchot et al. 1997; Freitas and Rocha 2000; Biassoni et al. 2001). Interestingly, a decreased pool of CD8⁺ T-lymphocytes and altered TCR repertoire are common features associated with HH (Reimao et al. 1991; Porto et al. 1994; Cardoso et al. 2001). It is tempting to suggest that these defects arise from the incapacity of HFE C282Y mutant cells to correctly process and display MHC-I at the cell surface.

MHC-I plays an inhibitory role in the shaping of the NK cells' response. An insufficient signaling by MHC-I-specific inhibitory receptors present on NK cells triggers their activation and cytotoxicity against cells that are either MHC-I negative or deficient (Biassoni et al. 2001). The decreased cell surface expression of MHC-I molecules in HFE mutant cells makes them suitable candidates for targeting by NK cells. There is evidence that down-regulation of MHC-I on resting lymphocytes was sufficient to make them susceptible to NK cell killing (Oberg et al. 2004). HFE itself does not inhibit or activate the activity of NK cells (Pascolo et al. 2005), but by impacting on MHC-I expression, HFE C282Y mutant cells may become particularly suitable targets of NK cytotoxic role.

With the noticeable exception of HFE, all the non-classical MHC-I molecules were shown to be directly involved in immunological processes (Braud et al. 1999). However, a growing amount of evidence has been questioning the validity of this assumption. Evidence for the involvement of HFE on a classical immunological mechanism was recently provided. Using transgenic mice, it was observed a direct recognition of human HFE by mouse TCR independently of HFE-bound peptides (Rohrlich et al. 2005). This new function of HFE is in agreement with the previous finding that TCR-delta knockout mice develop hepatic iron overload suggesting that cellular iron status might be transmitted to lymphocytes through HFE engagement with the TCR (Ten Elshof et al. 1999). Together with the impact of HFE C282Y on MHC-I expression, this finding constitutes the first molecular evidence for the participation of HFE in an iron-unrelated physiological event.

Collateral damages or selective interference? The UPR effect on the MHC-I expression

Some particular conditions, such as the synthesis of mutant proteins, flood the ER folding machinery and the ERAD components. To cope with this burst of ER clients, the cell triggers the UPR, which, employing an exclusive signaling pathway, enhances the levels of proteins that participate in folding and ERAD and attenuates protein synthesis (Schroder and Kaufman 2005c). By preventing HFE association with $\beta 2m$, the C282Y mutation causes the ER retention of HFE (Waheed et al. 1997; Pinto et al. 2007). Hypothetically, accumulation of this faulty protein in the ER proceeds until such point when its levels rise above threshold leading to the activation of the UPR. In fact, using a cell line model system genetically manipulated to express either HFE wt or HFE C282Y,

higher levels of specific UPR markers were observed in those cells expressing the mutant protein (Chapter 3). Significantly, *ex vivo* PBMCs from HFE C282Y homozygous HH patients recapitulated this result establishing the UPR activation as a putative modifier of HH, taking into account its decisive role on the progression of several other diseases.

Having reported the existence of MHC-I anomalies in HFE C282Y expressing cells, and considering the numerous intracellular events modified by the UPR signaling, it was tempting to consider that the UPR signaling pathway might mediate the HFE/MHC-I cross-talk. Actually, induction of the UPR, either pharmacologically or by the transfection of HFE C282Y, resulted in a significant down-regulation of the MHC-I cell surface expression. Moreover, inhibition of the ER stress response in cells expressing the HFE C282Y mutant protein led to the restoration of the MHC-I levels (Chapter 3). A strong link between the UPR and the MHC-I antigen presentation pathway defects was established. However, this association was found to prevail beyond the mere significance for the HH associated MHC-I defects. Regardless of the ER stress stimuli tested, it was noted that activation of the UPR consistently provokes a significant collapse of the MHC-I processing apparatus resulting in its cell surface expression decline (Chapter 3).

The broad impact of the UPR on distinct cellular events, alongside the complexity of the antigen presentation pathway, make the establishment of a mechanistic link connecting the ER stress response activation and the MHC-I impairment a rather complicated task. However, in light of some processes known to participate in both pathways, it is possible to envisage some plausible reasons for the MHC-I deficiency.

As part of the struggle to relief the folding pressure launched upon the ER, activation of the UPR promotes protein trafficking from and beyond this compartment through the induction of cargo receptors (Sato et al. 2002; Nyfeler et al. 2003). The lectin ERGIC-53, a cargo receptor for the ER export of proteins and its related protein VIP36 are known targets of the UPR signaling pathway (Nyfeler et al. 2003). The premature expulsion of ER clients in situations of stress may have a significant impact on proteins relying on a dedicated quality control mechanism to achieve a stable conformation. MHC-I molecules depend on the PLC to promote the assembly of low off-rate MHC-I/peptide complexes before their journey through the standard secretory pathway (Elliott and Williams 2005). This ensures that only complexes with superior stability are produced and delivered to the final destination. The extent of this peptide loading optimization is determined by the time spent by MHC-I complexes in the ER (Lewis and Elliott 1998) and this might be considerably shortened following UPR activation. It is therefore tempting to propose that, under stressful situations, the compulsory release of MHC-I/peptide complexes from the ER results in the production of volatile complexes that fail to reach the cell surface due to premature peptide dissociation. In agreement, it was observed that MHC-I ER egress occurs faster in HFE C282Y mutant cells than in the wt counterparts. Furthermore, thermostability and endocytosis assays revealed a premature MHC-I/peptide dissociation in HFE mutant cells, which resulted in an increased rate of endocytosis from the plasma membrane (Chapter 2).

Newly synthesized proteins that fail to acquire a native conformation due to errors in transcription, translation, folding, intracellular trafficking or assembly, are collectively referred to as DRiPs and, following proteasomal degradation, constitute the major font of antigenic peptides for MHC-I presentation (Yewdell et al.

1996) (Chapter 1 – Part I). It was previously observed that blocking protein synthesis with cycloheximide reduces peptide translocation by TAP and MHC-I expression (Reits et al. 2000; Schubert et al. 2000) by depleting the pool of rapidly degraded proteins such as DRiPs (Qian et al. 2006). In a cellular environment where the ER folding capacity is disrupted, cells are further burdened by the continuous production of protein. To mitigate this added stress, one of the strategies employed by the UPR signaling pathway is the attenuation of protein translation by phosphorylated eIF2 α (Harding et al. 1999) (Chapter 1 – Part III). However, the solid dependency of the antigen presentation pathway on fully operational transcription/translation machinery suggests a negative impact of the UPR on the MHC-I assembly. By inhibiting protein synthesis, the ER stress response might cease the most important source of MHC-I peptides, thus preventing its correct processing and cell surface expression.

Although not addressed, the involvement of several molecular chaperones (e.g. BiP, GRP94 and calreticulin) on the UPR progression may also indirectly perturb the folding and maturation of MHC-I molecules in the ER. Moreover a possible interference on more specific partners of MHC-I, like tapasin or TAP, would also account for the antigen presentation defects observed in ER stressed cells.

Stressing out the host to escape immune surveillance

Together with a sophisticated collection of immune evasion strategies, the interference on cellular homeostasis, namely the direct targeting of the ER function, is a characteristic of several viruses. Actually, triggering of the UPR signaling pathway was already described in a number of viral infected cells (Su et al. 2002; Tardif et al. 2002; Liu et al. 2004; Tirosh et al. 2005; Hegde et al. 2006). Although the reason for this is incompletely understood, it is attractive to propose that it may constitute one of the tools that viruses have developed to escape immune surveillance. The finding that an active UPR impairs MHC-I expression (Chapter 3) provides the rationale for this hypothesis. Modulation of the ER stress response would give viruses an additional method to disrupt antigen presentation by preventing the correct trafficking of MHC-I/peptide complexes to the cell surface.

Some previous studies have already insinuated an association between viral induction of the UPR and subversion of the MHC-I antigen presentation pathway on infected cells. ER stress-mediated interference on MHC-I assembly and cell surface expression was previously suggested to allow hepatitis C virus avoid detection and elimination by the immune system (Tardif and Siddiqui 2003). Similarly, it was observed that HCMV protein US11 provokes an UPR that may facilitate the degradation of MHC-I molecules (Tirosh et al. 2005). A more detailed characterization of this link and, particularly, of the HFE C282Y-associated UPR contribution for the establishment and progression of viral infections emerges as an attractive future challenge.

Possible implications of the UPR on the “*iron genes*” and HH

Following the UPR activation, several transcription factors known to control the expression of genes involved in protein folding/degradation are activated in an attempt to overcome the pressure exerted upon the ER

(Schroder and Kaufman 2005b). However, it is possible that transcription of genes not directly recognized as classical UPR targets might also be modulated. Herein, it is of particular significance to consider that the UPR signaling pathway could also aim genes involved in iron metabolism, thus contributing for the progression of HH itself. Supporting this hypothesis, analysis of 5' UTR reveals the presence of conserved transcription factor binding sites in classical "*iron genes*" like ferritin (Wasserman and Fahl 1997) or ferroportin (Table 1).

Table 1 - Ferroportin gene 5' UTR analysis. PubMed ID – DQ072115			
	Putative transcription factor binding sites:		
	XBP1 binding site	ERSE	CRE
Position	4302 - 4322	2215 – 2227 2979 – 2991 4229 – 4241 5465 – 5477	5743 – 5763
Core similarity*	1.000	1.000	1.000
Sequence	atgccctc ACGT ggcatttc	ctg CCAC cctcct cag CCAC ccagca cca CCAC gcctgg cag CCAC tctgct	tgggtgtgg TGAC gtatacct

XBP1 (X-Box binding protein 1); ERSE (ER stress element); CRE (cAMP responsive element), see Chapter 1 – Part III for details. The maximum core similarity of 1.0 is reached when the highest conserved bases of a matrix match exactly in the sequence. Data were obtained using the software MatInspector (<http://www.genomatix.de>).

Ferritin is one of the biochemical markers of iron overload. Elevated levels of this iron storage protein are commonly found in HH patients. Analysis of the promoter region revealed the existence of an ARE sequence on both the ferritin-H and -L genes (Wasserman and Fahl 1997). After the UPR activation, phosphorylation by PERK results in Nrf2 nuclear localization and activation of transcription through the ARE (Chapter 1 – Part III) (Venugopal and Jaiswal 1998; Nguyen et al. 2000). The finding that the transcription factor Nrf2 binds the ARE in the ferritin gene (Pietsch et al. 2003) constitutes a direct link between the UPR activation and increased ferritin transcription. This suggests the existence of a new pathway for the regulation of ferritin transcription, complementary to the iron-responsive element-binding proteins (IRE/IRP)-mediated control of ferritin mRNA translation (Hentze et al. 2004). In HH, both regulatory pathways would contribute to boost the production of ferritin: Nrf2 by promoting its transcription and the IRE/IRP by relieving the downstream mRNA translation blockage.

Ferroportin is an iron transporter involved in intestinal iron absorption and cellular iron release playing a determinant role in iron homeostasis (Donovan et al. 2005). In HFE C282Y homozygotes the ferroportin mRNA levels are significantly higher than in wt subjects (Bridle et al. 2003; Gleeson et al. 2007). The up-regulation of

FPN1 mRNA is indicative of an IRE/IRP-independent regulatory mechanism. Moreover, in HH, the observed increase in ferroportin levels despite the high degree of iron loading antagonizes the expected IRE/IRP-mediated posttranscriptional inhibition. In fact, it was previously suggested that ferroportin levels are regulated transcriptionally rather than by the IRE/IRP control of mRNA translation (Zoller et al. 2002). Hence, it is attractive to consider that transcription factors generated by the UPR activation in HFE mutant cells promote ferroportin transcription through one of the identified putative binding sites (Table 1).

Ferroportin is posttranslationally regulated by hepcidin, which causes its internalization and degradation, thereby blocking iron flow into plasma (Nemeth et al. 2004). Hepcidin is a small peptide hormone secreted by the liver into the circulation. Its transcription is strictly correlated with the iron needs: synthesis increases if iron stores are high and decreases when iron stores are depleted (Nemeth and Ganz 2006).

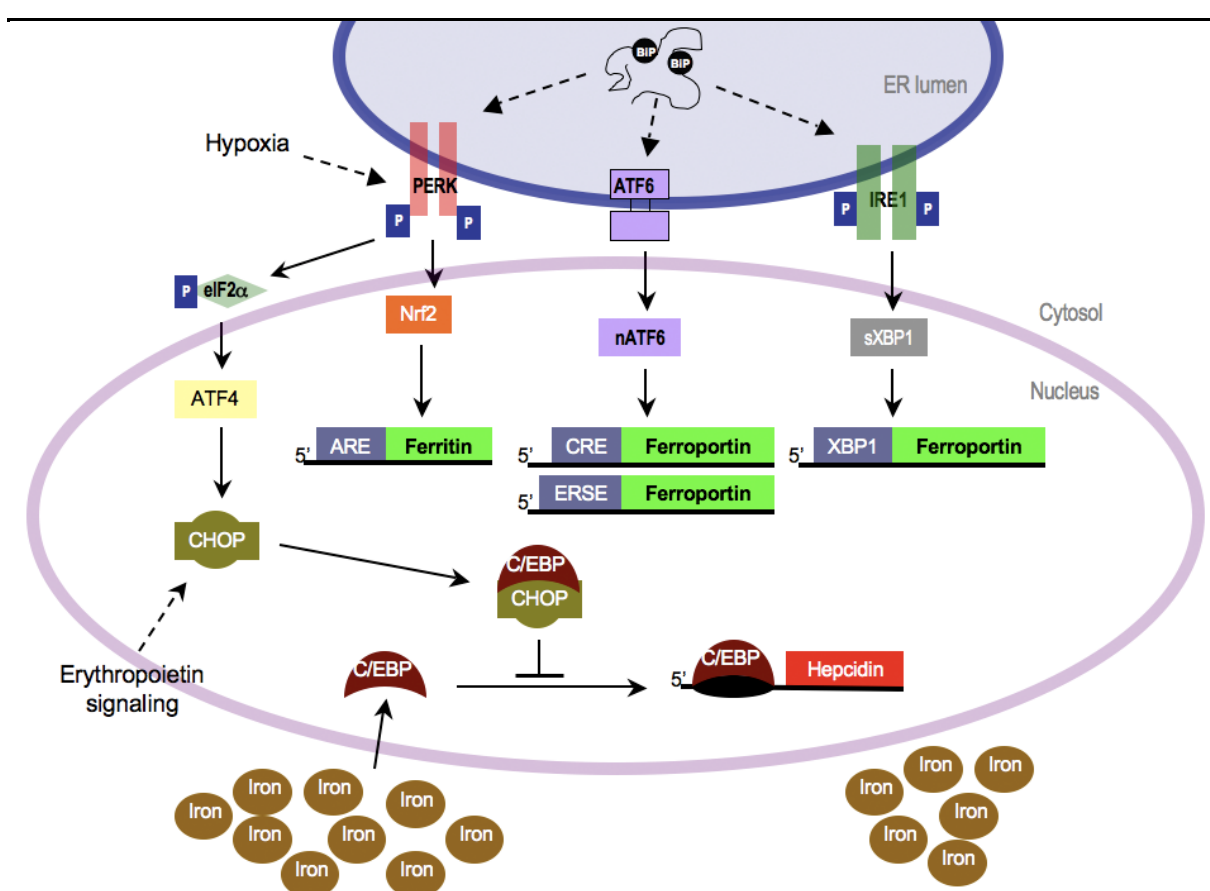


Figure 1 – Proposed model for the interference of the UPR signaling pathway on the expression of “iron genes”. HFE C282Y accumulation in the ER initiates the UPR. Activation of PERK promotes CHOP transcription. As a result, C/EBPα-driven hepcidin expression is abrogated by CHOP, leading to low levels of hepcidin transcription despite the increased iron loading. PERK activation promotes transcription of ferritin through Nrf2 binding to the antioxidant-responsive element (ARE) sequence. Activation of ATF6 and IRE1 results in the activation of transcription factors that bind to cyclic AMP responsive element (CRE), ER stress response element (ERSE) or XBP-1 binding site (XBP1) (see Table 1 and text for details) on ferroportin promoter increasing its transcription. Hypoxia and erythropoietin increase CHOP levels and down regulate hepcidin expression, possibly through C/EBPα inhibition. See text for details.

C/EBPα was previously identified as a potent activator of the hepcidin promoter. Iron overload leads to increased C/EBPα, which in turn stimulates hepcidin transcription (Courselaud et al. 2002). In HH, given the

degree of iron loading, one would expect a significant increase in hepcidin levels through the C/EBP α pathway. However, hepcidin is improperly low in HH patients (Bridle et al. 2003). CHOP, one of the UPR targets (Chapter 1 – Part III), is known to dimerize with C/EBP α acting as a dominant-negative inhibitor of gene transcription (Ron and Habener 1992). Interestingly, increased levels of CHOP were observed in HH patients' cells (Chapter 3), suggesting that the transcriptional repression through inhibition of C/EBP α might prevent enhanced hepcidin synthesis in response to iron overload.

Besides iron deficiency, hypoxia also leads to diminished hepcidin production. It was previously reported that exposure to a low oxygen atmosphere causes a decrease in hepcidin mRNA levels through an unrevealed mechanism (Nicolas et al. 2002). Adaptation of cells to hypoxic stress requires activation of the UPR (Koumenis et al. 2002). More specifically, a successful response to hypoxia demands an intact PERK/eIF2 α /ATF4 signaling pathway (Fels and Koumenis 2006), which results in the induction of the downstream gene CHOP (Harding et al. 2000).

Similarly, increased CHOP levels were observed after treatment with erythropoietin (Coutts et al. 1999). More recently it was shown that stimulation with erythropoietin decreases hepcidin levels (Fein et al. 2007). Therefore, it is tempting to suggest that CHOP is specifically involved in the transcriptional repression of hepcidin during hypoxia and after stimulation with erythropoietin through C/EBP α inhibition, as hypothesized for HH.

The UPR and disease progression: HFE C282Y as a risk factor for diabetes

Recent advances on the understanding of UPR signaling mechanisms have led to important new insights into the diverse cellular and physiologic processes that are regulated by this ER stress response. Designed to protect cells from insults on the protein folding and secretory capacity, it is manifest that the involvement of the UPR in several pathological conditions influences their progression. On the other hand, even in the absence of any detrimental ER stress stimulus, an intact UPR signalling pathway is crucial for a number of physiological events (Chapter 1 – Part III).

Previous studies have demonstrated that the UPR is a central feature of peripheral insulin resistance and type 2 diabetes (Ozcan et al. 2004). It was found that obesity contributes to the development of type 2 diabetes by causing ER stress, which suppresses the insulin-receptor signaling through hyperactivation of JNK and subsequent serine phosphorylation of insulin receptor substrate-1. Accordingly, the observation that HH patients have an active UPR (Chapter 3) should place HFE C282Y carriers at higher risk for type 2 diabetes by developing insulin resistance (Figure 2). In fact, several studies have reported that the C282Y mutation was more frequent in type 2 diabetic patients than in healthy control subjects (Fuchs et al. 2002).

Dysfunction of insulin secretion is a key element in the pathophysiology of type 1 diabetes. The progressive failure of pancreatic β cells to secrete sufficient amounts of insulin to compensate for the insulin resistance culminates in hyperglycemia. Proinsulin synthesis mechanisms demand the physiological contribution of the UPR (Chapter 1 – Part III). Fluctuations of glucose levels shut the PERK-mediated inhibition of proinsulin

mRNA translation on or off (Scheuner et al. 2001). Low glucose impairs protein glycosylation and ATP production, which disturbs protein folding in the ER causing the UPR activation. Increasing glucose levels then restore the ER function and terminate the stress response. This delicate balance between activation and inhibition of the UPR anticipates the existence of proinsulin synthesis defects in HFE C282Y individuals. Theoretically, a chronic UPR, like the one obtained by the continuous expression of a defective protein (e.g. HFE C282Y), would bias the system towards poor proinsulin production and eventually to pancreatic β cell death through UPR-mediated apoptosis (Figure 2). The Akita mouse, in which β cell death resulting from the UPR activation culminates in diabetes (Chapter 1 - Part III), illustrates the pathological potential of a chronic ER stress response. Moreover, since compelling evidence strongly suggests that protein aggregates are cytotoxic (Caughey and Lansbury 2003), the observation that HFE C282Y proteins convene to form these intracellular oligomeric species (Chapter 4) may also contribute to β cell loss of function and insulin deficiency.

The possible implication of the HFE C282Y-associated UPR on both the insulin resistance mechanisms and the defective insulin secretion by β cells warrants further investigation on the prevalence of the C282Y mutation amongst diabetic patients.

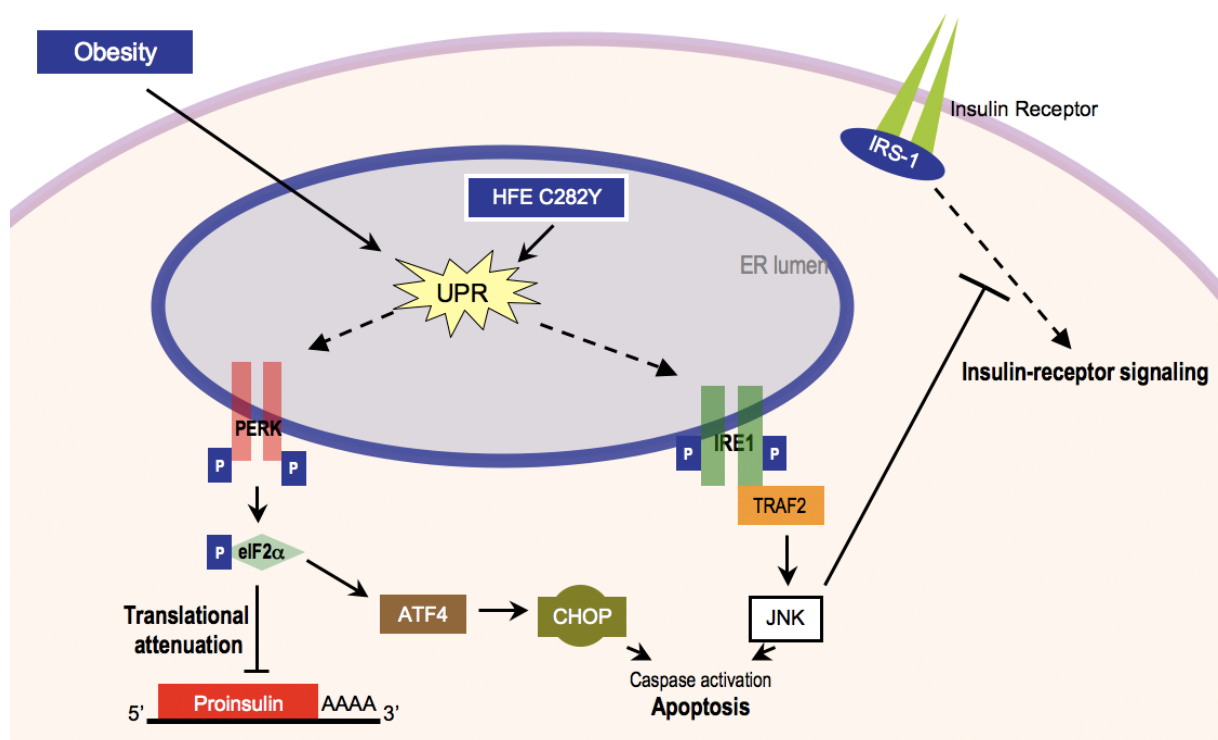


Figure 2 – Model for the impact of the UPR in diabetes. Obesity and HFE C282Y induce the unfolded protein response. Activation of PERK leads to eIF α phosphorylation that blocks translation of proinsulin even under higher glucose levels. Following the UPR induction, IRE1 activation suppresses the insulin-receptor signaling through hyperactivation of JNK. CHOP and JNK activation can culminate in pancreatic β cell apoptosis. (IRS-1: Insulin receptor substrate 1).

Pharmacological shaping of the ER stress response

By mimicking cellular molecular chaperones, which are ubiquitous stress-induced proteins, chemical and pharmacological chaperones have been found to improve the ER folding capacity and facilitate the trafficking of mutant proteins by stabilizing their conformation (Welch and Brown 1996). The therapeutic potential of these small molecular weight compounds, such as tauroursodeoxycholic acid (TUDCA) and sodium 4-phenylbutyrate (4PBA), in ameliorating the severity of a number of protein-misfolding diseases was already demonstrated. 4PBA was previously shown to increase the trafficking of a mutant cystic fibrosis transmembrane regulator (Rubenstein et al. 1997) and to enhance the secretion of the mutant α 1- antitrypsin Z protein (Burrows et al. 2000). Endogenous bile acids derivatives, such as TUDCA, can also modulate ER function protecting from UPR induction and ER stress-induced apoptosis (Xie et al. 2002; Ozcan et al. 2006). Recently, these chemical chaperones were shown to improve glucose tolerance and insulin action in a mouse model of type 2 diabetes (Ozcan et al. 2006). Moreover, TUDCA is already applied as a hepatoprotective agent in humans with cholestatic liver diseases (Poupon et al. 1999; Kaplan and Gershwin 2005), while 4PBA has been in clinical use in trials for treatment of thalassemia (Collins et al. 1995).

The finding that an UPR is activated in HH (Chapter 3) has motivated the investigation of the effect of these promising pharmacologically active compounds in the modulation of this stress response. In fact, despite the failure to rescue the mutant protein from ERAD and to restore its cell surface expression, both TUDCA and 4PBA impeded the UPR activation in HFE C282Y expressing cells (Chapter 4). Moreover, chemically enhancing the ER function using 4PBA also prevented the formation of intracellular protein aggregates by facilitating the degradation of HFE C282Y. Since the connection between the UPR and HH is shown for the first time here, additional studies are needed to elucidate the contribution of this ER stress response for the pathophysiology of the disease. A clear elucidation of its impact on the “*iron genes*”, its putative contribution for the progression of diseases like diabetes or the physiological significance of its associated MHC-I defects will certainly dictate the value of preventing the stimulation of the UPR using chemical chaperones. Nevertheless, the confirmed applicability of these compounds to avoid the ER stress response and of 4PBA in particular to prevent the formation of putatively toxic intracellular aggregates in HFE C282Y mutant cells stimulates further research aiming at exploring their therapeutic application in HH.

Future perspectives and concluding remarks

The journey towards the ultimate characterization of HFE as a multitask protein is underway. A protein previously thought to participate exclusively in iron homeostasis is now revealing other hidden faces. The results included in the present thesis, together with recent findings from other independent groups, place the immunological system as a particularly fit candidate to use the services of HFE. But the effect might be broader: the commitment of the UPR to a number of distinct physiological and pathological events boosts the interest on the C282Y mutation. Nevertheless, several questions remain unanswered justifying the need for further studies.

The observation of MHC-I defects in HFE C282Y mutant cells (Chapter 2) motivates additional investigation in order to understand their physiological relevance and their mechanistic origin. Evaluation of the MHC-I impairment's putative contribution to the immunological abnormalities associated with HH and its relevance to the mechanisms of defense against pathogens such as virus, arise as a particularly significant task.

The discovery of the HFE C282Y-associated UPR established a link between this mutant protein and the MHC-I antigen presentation pathway (Chapter 3). Analysis of the impact of the ER stress response on other cellular processes, namely those involved in iron homeostasis, emerges as a research priority in the attempt to find a parallel between the UPR and the pathophysiology of HH. The modulation of "*iron genes*" like ferroportin, ferritin or hepcidin should be further explored in light of the amount of evidence pointing to their putative susceptibility to experience transcriptional or translational regulation by the UPR signaling pathway.

Several studies have previously aimed at drawing a correlation between HH and the progression of other diseases. It is now pertinent to dissect the putative contribution of both the HFE C282Y-associated UPR and the intracellular HFE C282Y-aggregates (Chapter 4) as a risk factor for the onset of pathological conditions like diabetes. The outcome of these experiments will then dictate the relevance of testing the *in vivo* therapeutic potential of pharmacological chaperones like TUDCA or 4PBA.

The first steps are carefully but enthusiastically taken and new doors were opened. Now, a "*brave new world*" builds up alongside HFE and the role of its C282Y mutation. Time will certainly reveal whether this enterprise contributed to a decisive and profitable understanding of HH.

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List of abbreviations

4PBA, sodium 4-phenylbutyrate;
ARE, antioxidant response element;
ATF6, activating transcription factor-6;
nATF6, nuclear targeted and transcriptionally active fragment of ATF6;
BBF2H7, BBF2 human homolog on chromosome 7;
 β 2m, β 2-microglobulin;
BiP, H chain binding protein;
CHOP, C/EBP homologous protein;
CRE, cyclic AMP responsive element;
DRiPs, defective ribosomal products;
EDEP, ER degradation-enhancing 1,2 mannosidase-like protein;
ER, endoplasmic reticulum;
ERAD, ER-associated degradation;
ERSE, ER stress response elements;
GRP94, glucose regulated protein 94;
HC, heavy chains;
HCMV, human cytomegalovirus;
HH, Hereditary Hemochromatosis;
HIV, human immunodeficiency virus;
HSV, herpes simplex virus;
IRE1, inositol-requiring kinase 1;
IRE/IRP, iron-responsive element-binding proteins;
JNK, c-Jun N-terminal kinase;
KSHV, Kaposi sarcoma-associated herpes virus;
MHC-I, class I major histocompatibility complex;
NK cells, natural killer cells;
PDI, protein disulfide isomerase;
PERK, RNA-activated protein kinase-like ER kinase;
PLC, peptide loading complex;
Poly(Q), polyglutamine;
QC, quality control;
RNase, endoribonuclease;
TAP, transporter associated with antigen presentation;
TCR, T-cell receptor;
TfR1, transferrin receptor 1;

TUDCA, tauroursodeoxycholic acid;

UGGT, UDP-glucose:glycoprotein glucosyltransferase;

UPR, unfolded protein response;

wt, wild type;

XBP-1, X box-binding protein-1;

sXBP-1, spliced form of XBP-1;

Summary

In contrast with the other members of the large family of major histocompatibility complex class I (MHC-I)-like proteins, whose participation in immunological processes is a common feature, since its discovery, HFE has been referenced to as playing a decisive role in the regulation of iron mobilization by cells. The link with Hereditary Hemochromatosis (HH) has characterized the rather short life of this non-classical MHC-I protein and interest in HFE has relied mainly on its participation in the control of iron metabolism, namely through its association with the Transferrin Receptor I. Paradoxically however, the advances in the characterization of the disease provided the clues for an extended function of HFE. The identification of immunological anomalies associated with HH has highlighted the need for a detailed study of the immune system in these patients. One characteristic of these anomalies is the involvement of an individual subset of lymphocytes: the CD8⁺ T-lymphocytes. The key role exerted by MHC-I in these cells strongly suggested that deficiencies in this protein's function could be at the origin of the immunological defects observed in HH patients. To evaluate the putative involvement of HFE on such processes, specifically by investigating the impact of its C282Y mutation on the MHC-I antigen presentation pathway, constituted the original aim of the present thesis.

A comprehensive study of the MHC-I intracellular trafficking in peripheral blood mononuclear cells (PBMCs) of HH patients carrying the HFE C282Y mutation revealed the existence of several flawed events that culminated in a decreased cell surface expression of MHC-I molecules. These findings constituted the first evidence for the existence of a cross-talk between the C282Y mutation in HFE and the MHC-I antigen presentation pathway.

The current knowledge of the intracellular fate of HFE C282Y together with the fact that cells have evolved highly specific signaling pathways to cope with the accumulation of unfolded or misfolded proteins fuelled the interest to investigate the contribution of these stress responses to the intracellular processing defects of MHC-I molecules observed in HH. In both a cell line model system genetically modified to express either HFE wt or C282Y and in PBMCs obtained from HH patients, it was observed that the mutant protein triggers an unfolded protein response (UPR) that led to a decrease in MHC-I cell surface expression. This defect in MHC-I was mimicked by inducing the UPR with other unrelated UPR stimuli and corrected in HFE C282Y expressing cells by blocking this signaling pathway. More than merely establishing a link between the HFE C282Y mutation and the MHC-I anomalies, these results indicate that a similar effect may occur in other diseases where an active UPR is found. Neurodegenerative diseases, diabetes, cancer and viral infections arise as particularly significant candidates, both by the existence of an UPR and by the targeting of the MHC-I pathway already observed in some of them (namely upon viral infections).

Some compounds, collectively referred to as chemical chaperones, have the ability to facilitate the trafficking of mutant proteins and to inhibit the UPR induction and the ER stress-induced apoptosis, thus providing an interesting therapeutic approach for a large number of diseases. The ultimate aim of the present thesis consisted in evaluating the effect of chemical chaperones on both the HFE C282Y trafficking and UPR activation. Treatment of HFE C282Y expressing cells with tauroursodeoxycholic acid (TUDCA) or with sodium 4-

phenylbutyrate (4PBA) successfully hampered the magnitude of the UPR. Analysis of the effect of these chemical chaperones on the intracellular trafficking of the mutant HFE revealed that while failing to restore its cell surface expression, 4PBA successfully prevented the intracellular protein aggregation that was found to occur in cells expressing HFE C282Y. This finding offers a potential new strategy for therapy designed to prevent the potential toxicity of the aggregates.

Primarily designed to investigate the immunological role of HFE mutations, the conclusion of this thesis allowed to uncover the existence of an intersection between the cell biology of HFE C282Y and the MHC-I antigen presentation pathway, but also to extend the significance of the results to other diseases that share some of the cellular mechanisms, namely the UPR, with HH. In addition, the data obtained with the chemical chaperones open new perspectives on the use of these compounds as a therapeutic approach for HH.

Resumo

Ao contrário dos restantes membros da família de proteínas homólogas ao complexo major de histocompatibilidade classe I (MHC-I), cuja participação em processos imunológicos é uma característica comum, desde a sua descoberta, o HFE tem sido referenciado como desempenhando um papel na regulação da mobilização de ferro pelas células. A ligação com a Hemocromatose Hereditária (HH) tem caracterizado a curta vida desta proteína MHC-I não-clássica e o interesse no HFE tem residido principalmente na sua participação no controlo do metabolismo do ferro, nomeadamente através da sua ligação ao Receptor da Transferrina I. Curiosamente, foram os avanços conseguidos na caracterização da HH que forneceram as pistas para uma função adicional do HFE. A identificação de anomalias imunológicas associadas à HH justificam a necessidade de se efectuar um estudo detalhado do sistema imunitário destes doentes. Uma característica destas anomalias é o envolvimento de uma população específica de linfócitos: os linfócitos T CD8+. O papel determinante exercido pelo MHC-I nestas células sugeriu que deficiências nesta proteína poderiam estar na origem das anomalias detectadas nos doentes de HH. A avaliação do envolvimento do HFE nestes processos, nomeadamente investigando o impacto da mutação C282Y na via de apresentação de antígenos pelo MHC-I, constituíram o principal objectivo desta tese.

Um estudo exaustivo do tráfego intracelular do MHC-I em células mononucleadas de sangue periférico (PBMCs) de doentes de HH portadores da mutação C282Y no HFE revelou a existência de vários eventos imperfeitos que culminaram numa reduzida expressão de MHC-I na superfície destas células. Estas observações constituem a primeira evidência para a existência de uma intercepção entre a mutação C282Y no HFE e a via de apresentação de antígenos pelo MHC-I.

Os avanços na caracterização do processamento intracelular do HFE C282Y bem como o facto das células possuírem a capacidade de activar vias de sinalização específicas que lhes permitem responder à acumulação de proteínas com uma conformação deficiente motivou o interesse na investigação da contribuição destes mecanismos de resposta ao stress para os defeitos no processamento intracelular do MHC-I observados na HH. Utilizando uma linha celular modificada geneticamente para expressar o HFE wt ou o HFE C282Y e também PBMCs obtidos através do sangue de doentes de HH, foi verificado que a proteína mutante activa uma resposta ao stress provocado pela acumulação de proteínas incapazes de adquirir uma conformação correcta (UPR) que por sua vez leva à diminuição da expressão de MHC-I à superfície das células. Este defeito no MHC-I foi igualmente observado após a indução da UPR com outros estímulos independentes e corrigido se em células contendo o HFE C282Y a UPR fosse bloqueada. Mais do que estabelecer uma ligação entre o HFE C282Y e as anomalias no MHC-I, estes resultados indicam que um efeito similar poderá ter lugar noutras doenças em que a UPR está activa. Doenças neurodegenerativas, diabetes, cancro e infecções virais afiguram-se como candidates particularmente significativos.

Determinados compostos, designados chaperones químicos, têm a capacidade de facilitar o tráfego intracelular de proteínas mutantes e de inibir a activação da UPR, constituindo deste modo uma interessante

abordagem terapêutica para um vasto número de doenças. O objectivo final desta tese consistiu em avaliar o efeito dos chaperones químicos no tráfego do HFE C282Y e na UPR induzida por esta proteína mutante. O tratamento de células que expressam HFE C282Y com ácido tauroursodeoxicólico (TUDCA) ou 4-fenilbutirato de sódio (4PBA) revelou ser eficaz em atenuar a UPR. A análise do efeito destes chaperones químicos no tráfego intracelular do HFE mutante mostrou que embora a expressão à superfície da proteína não fosse restaurada, o 4PBA foi eficaz em prevenir a formação dos agregados proteicos intracelulares que se observaram em células que sintetizavam o HFE C282Y. Esta descoberta oferece uma nova estratégia terapêutica desenhada para prevenir a possível citotoxicidade destes agregados.

Originalmente concebida para investigar o papel imunológico das mutações do HFE, os resultados apresentados nesta tese permitiram desvendar a existência de uma intercepção entre o HFE C28Y e a via de apresentação de antígenos pelo MHC-I, sendo também possível sugerir que os resultados obtidos poderão ter algum significado no contexto de outras doenças que, tal como a HH, possuam uma UPR activa. Finalmente, os dados aqui apresentados abrem novas perspectivas em relação ao uso dos chaperones químicos numa possível estratégia terapêutica para a HH.

Résumé

Contrastant avec les autres membres de la grande famille de protéines homologues du complexe majeur d'histocompatibilité classe I (MHC-I), qui participent ordinairement en procès immunologiques, HFE a été, après sa découverte, identifié comme une protéine avec un rôle décisif dans la régulation de la mobilisation du fer. La liaison avec l'Hémochromatose Héréditaire (HH) a caractérisé la brève vie de cette protéine MHC-I non-classique, l'intérêt en HFE s'appuyant surtout à ça participation dans le contrôle du métabolisme du fer, nommément par l'association avec le Récepteur de Transferrine I. Paradoxalement, pourtant, les progrès dans la caractérisation de la maladie ont fourni les pistes pour une plus vaste fonction de l'HFE. L'identification des anomalies immunologiques associés à l'HFE a souligné la nécessité d'une étude plus détaillée du système immunitaire de ces patients. Caractéristique de ces anomalies c'est l'engagement d'une population spécifique de lymphocytes : les lymphocytes T CD8+. Le rôle déterminant de l'MHC-I dans ces cellules a suggéré que déficiences dans cette protéine pût être à l'origine des anomalies détectées dans les malades de HH. L'évaluation de l'engagement de HFE dans ces procès, nommément par l'investigation de l'impact de la mutation C282Y dans la voie de présentation d'antigènes par l'MHC-I, a été le principal objectif de cette thèse.

Une étude détaillée du trafic intracellulaire du MHC-I dans des cellules mononuclées du sang périphérique (PBMCs) de malades de HH portant la mutation C282Y dans HFE a révélé l'existence de plusieurs événements imparfaits qui ont culminé dans une expression réduite de MHC-I à la surface de ces cellules. Ces observations constituent la première évidence de l'existence d'une interception entre la mutation C282Y dans l'HFE et la voie de présentation d'antigènes par l'MHC-I.

Les progrès dans la caractérisation du processus intracellulaire du HFE C282Y, bien comme le fait que les cellules possèdent la capacité d'activer voies de signalisation spécifiques, qui leur permettent de répondre à l'accumulation de protéines avec une conformation déficiente, a poussé l'intérêt dans la recherche de la contribution de ces mécanismes de réponse au stress pour les anomalies dans le processus intracellulaire du MHC-I observé dans la HH. En utilisant une lignée cellulaire modifiée génétiquement pour exprimer l'HFE wt or l'HFE C282Y et aussi des PBMCs obtenus du sang des malades de HH, il a été vérifié que la protéine mutante active une réponse au stress entraîné par l'accumulation de protéines incapables d'acquérir une conformation correcte (UPR) qui a son tour, origine la diminution de l'expression du MHC-I à la surface des cellules. Cette anomalie dans l'MHC-I a été aussi observée après l'induction de l'UPR avec d'autres stimuli indépendants et corrigés si, en cellules contenant l'HFE C282Y, l'UPR fût bloquée. Plus qu'établir une liaison entre l'HFE C282Y et les anomalies dans l'MHC-I, ces résultats indiquent qu'un effet pareille pourra avoir lieu dans d'autres maladies ou l'UPR est active. Des maladies neurodégénératives, diabète, cancer, et les infections virales semblent des candidates particulièrement significantes.

Quelques composées nommées des chaperonnes chimiques, ont la capacité de faciliter le trafic intracellulaire de protéines mutantes et d'inhiber l'activation de l'UPR, constituant, donc, une approche thérapeutique intéressante pour un large nombre de maladies. L'objectif final de cette thèse a été l'évaluation de

l'effet des chaperonnes chimiques dans le trafic du HFE C282Y et dans l'UPR induite par cette protéine mutante. Le traitement des cellules qui exprime HFE C282Y avec l'acide tauroursodéoxicolique (TUDCA) ou 4-fenylbuthyrate de sodium (4PBA) a montré son efficacité dans l'atténuation de l'UPR. L'analyse de l'effet de ces chaperonnes chimiques dans le trafic intracellulaire du HFE mutante a montré que bien que l'expression de la protéine à la surface n'a pas été restaurée, le 4PBA a été efficace dans la prévention de la formation d'agrégats protéiques intracellulaires qui ont été observés en cellules qui synthétisaient l'HFE C282Y. Cette découverte offre une nouvelle stratégie thérapeutique dessinée pour prévenir la possible toxicité de ces agrégats.

Dessinée originalement pour investiguer le rôle immunologique des mutations de l'HFE, la conclusion de cette thèse a permis de révéler l'existence d'une intersection parmi la biologie cellulaire de l'HFE C282Y et la voie de présentation d'antigènes du MHC-I, mais aussi d'étendre la signification des résultats à autres maladies qui partagent quelques des mécanismes cellulaires, notamment l'UPR, avec HH. En plus, les données obtenues avec les chaperonnes chimiques ouvrent des nouvelles perspectives dans l'application de ces composés comme un abordage thérapeutique pour l'HH.

