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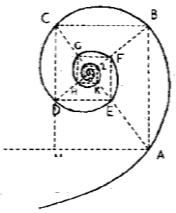

SCUOLA DI DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE
Curriculum di Oncologia Molecolare
Ciclo XXII

TESI DI DOTTORATO

The proteostasis of the endoplasmic reticulum and the activation of the unfolded protein response pathway *in vivo*

Il dottorando
Dr.ssa Luigina Tagliavacca
Matr. R07127

Il Tutore
Prof. Michele Samaja

Il Direttore
Prof.ssa Maria Luisa Villa

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ABSTRACT

Background. The protein components of eukaryotic cells face acute and chronic challenges to normal folding, refolding and function owing to a constant barrage of physical, metabolic and environmental stresses. Eukaryotic protein homeostasis, or proteostasis, enables healthy cell adaptation during development and protects against aging and diseases. Proteostasis refers to controlling the concentration, conformation, binding interaction and location of individual proteins making up the proteome by readapting the innate biology of the cell, often through transcriptional and translational changes. The endoplasmic reticulum (ER) responds to the accumulation of unfolded proteins in its lumen (ER stress) by activating intracellular signal transduction pathways, cumulatively called the unfolded protein response (UPR). The UPR activation triggers an extensive transcriptional and translational response, which adjusts the ER protein folding capacity according to needs. As such, the UPR constitutes one of the signaling pathways that regulates the capacity and composition of the proteostasis network according to the changing of the ER folding capacity.

Previous work. My initial work, described in **chapter 1.2.1** shows that the level of cellular energy is important for protein folding and disaggregation and thus can affect folding and repair processes.

The proteostasis network is not only highly adaptable, enabled by the influence of multiple cell stress signaling pathways, but also can be quite distinct in each cell type. In **chapter 1.2.2,** by using as a cellular model the plasma cells differentiation, I could underline the role of the UPR signaling and proteasomal degradation in orchestrating the architectural and functional changes of the cells and balancing the proteostasis capacity.

Recent studies suggest that lack/decrease of O_2 perturbs the ER homeostasis. Indeed the ER has emerged as a cellular compartment that depends on O_2 for oxidative folding of secretory and transmembrane proteins and that mediates the O_2 signaling that is important for the survival and function of hypoxic cells. *In vitro* studies have shown that hypoxia triggers the UPR. However, *in vivo* and *in vitro* situations are indeed likely to diverge substantially with respect to parameters such as metabolic activity, O_2 utilization and cell division rates, features that are predicted to vary the cell sensitivity to ER stress. Thus, despite a recognized role for hypoxia on UPR, few data exist on the effects of hypoxia in various organs *in vivo*.

Aims. My study includes three aims: First, we will test if the hypoxic stress *in vivo* acts as a modifier that affects the activation of specific branches of the UPR in different tissues. Second, to get a better insight into the role of the UPR during low oxygen availability in tissues, we will test whether the UPR activation depends from the severity of the hypoxic stress. Third, we aim at delineating signaling circuits that control the capacity and composition of the proteostasis network through transcriptional and post transcriptional mechanisms to balance the ER homeostasis

Results. I analyzed the effect of the hypoxic stress on the proteostasis network. Changes in O_2 levels alter the ability of the cells to handle the proteostasis load with some differences between the cells type studied. Hepatocytes and myocytes

respond to hypoxia by increasing their degradation activity as to increase the proteostasis capacity. While the hepatocytes activate an UPR-dependent apoptosis and are able to balance between apoptotic death and protein synthesis, in the myocytes the protein synthesis remains sustained under low oxygen availability while the UPR –dependent apoptosis could not be detected.

Conclusion. This studies underlined several features of the ER- proteostasis. First, the proteostasis network is adaptable and able to fine tune the UPR signaling pathway in response to stress. Second, different cells have varying proteostasis capacities reflected in the composition and concentrations of their proteostasis components. Third, within a given cell type, the proteostasis does not possess significant excess capability, rater it is finely tuned and offers just enough facility for the protein folding load. Therefore, by setting the proteostasis boundary as a threshold for generating folded and functional proteins, the proteostasis network can create and maintain functional proteins in response to the local environment

RIASSUNTO

Introduzione. Le proteine delle cellule eucariotiche devono affrontare continue difficolta' durante il processo di ripiegamento conformazionale ed espletamento delle loro funzioni biologiche dovuti a stress cellulari di tipo fisico, metabolico e ambientale.
L'omeostasi delle proteine, detta proteostasi, permette l'adattamento cellulare durante lo sviluppo e protegge la cellula dall'invecchiamento e dallo stress. Il termine proteostasi si riferisce al controllo della concentrazione, conformazione, interazione e localizzazione delle proteine facenti parte del proteoma per permettere alla cellula di adattarsi alle mutate condizioni attraverso cambiamenti trascrizionali e traduzionali. Il reticolo endoplasmico (ER) risponde all'accumulo delle proteine misfoldate nel proprio lume (ER stress) attivando segnali intracellulari complessivamente chiamati Unfolded Protein Response (UPR). L'attivazione dell' UPR induce una risposta sia trascrizionale che traduzionale che mira ad aumentare la capacita' di ripiegamento delle proteine dell'ER per commensurarla alle nuove necessita' cellulari. In questo modo la cellula adegua la sua capacita' di sintesi e ripiegamento delle proteine secretorie a seconda delle proprie necessita'.
Lavoro iniziale. Il lavoro iniziale, descritto nel capitolo 1.2.1 dimostra che il livello di energia intracellulare è importante per il corretto ripiegamento proteico e per il recupero di proteine aggregate per cui influenza i processi sia di ripiegamento che di riparazione. L'insieme delle proteine che regolano l'omeostasi cellulare è molto adattabile, grazie alla presenza di diverse vie di segnale, e possiede caratteristiche distinte per tipi cellulari. Usando come modello la differenziazione delle plasma cellule nel ho potuto delineare il ruolo dell'UPR e della degradazione proteasomale nella regolazione dei cambiamenti strutturali e funzionali. (capitolo 1.2.2)
Studi recent suggeriscono che la mancanza o ridotta concentrazione cellulare di ossigeno modifica la proteostasi dell ER. Infatti il processo di ripiegamento ossidativo delle proteine transmembrana e secretorie dipende dalla concentrazione di ossigeno intracellulare e segnali cellulari vengono attivati in situazioni di ridotta concentrazione di O_2 importanti per il processo di sopravvivenza delle cellule ipossiche. Studi condotti in vitro hanno dimostrato che l'ipossia attiva l'UPR. Tuttavia analisi condotte in vitro e in vivo possono differire rispetto alcuni parametri come l'attività metabolica, l'utilizzo di O2 e la divisione cellulare, caratteristiche che variano la capacità delle cellule di gestire lo stress. Per cui le situazioni in vitro possono essere diverse da quelle in vivo.
Scopo. Il mio studio include 3 ipotesi. Primo analizzerò se lo stress ipossico in vivo attiva l'UPR o parte di esso in diversi tessuti. Secondo testerò se l'attivazione dell'UPR dipende dall'intensita' dello stress. Terzo mi propongo di delineare le vie di segnale che controllano la capacita' e la composizione dell'insieme di proteine che governano l'omeostasi cellulare.
Risultati. Ho analizzato l'effetto dell'ipossia sulla proteostasi del reticolo. Le cellule rispondono in modo diverso alle alterate disponibilita' di ossigeno cellulare a seconda della loro capacita' di adeguamento alla alterata omeostasi delle proteine. Sia gli epatociti che i miociti cardiaci aumentano l'attivita' di degradazione proteica

durante l'ipossia in modo da aumentare le capacita' proteostatiche. Inoltre gli epatociti attivano sia segnali di attenuazione della sintesi proteica che di apoptosis.
Conclusione. Questi studi hanno permesso di individuare diverse caratteristiche del processo omeostatico dell'ER. Primo, la proteostasi delle proteine e' adattabile e in grado attivare processi di UPR in seguito a stress. Secondo, le cellule possiedono diverse capacita' proteostatiche a seconda della loro funzione nell'organismo. Terzo, le cellule una limitata capacita' di ripiegare le proteine in eccesso in modo corretto. Quindi le cellule possiedono una specifica delimitata capacita' proteostatica tale da consentirne il funzionamento in risposta all'ambiente circostante.

Contents

LIST OF ABBREVIATIONS

ATF4 Activating Transcription factor 4
ATF6 Activating Transcription factor 6
BiP Immunoglobulin Bindin Protein, known as GRP78
CREBH Cyclic AMP Response Element Binding Protein Hepatocyte
eIF2 Eukaryotic Initiation Factor 2
ER- Endoplasmic Reticulum
4EBP1 Eukaryotic Initiation Factor 4E binding protein 1
ERAD ER-Associated Degradation
GADD153 Growth Arrest DNA Damage-Inducible Protein, known as CHOP
GADD34 Growth Arrest DNA Damage-Inducible Protein
HIF1 Hypoxia Inducible Factor
ISR Integral Stress Response
IRE1 Inositol Requiring Protein 1
JNK c-Jun N-terminal Kinase
mTOR mammalian target of Rapamycin
PDI Protein Disulphide Isomerase
PERK PKR-like Endoplasmic Reticulum Kinase
PKR Double-stranded RNA-activated Protein Kinase
UPR Unfolded Protein Response
VEGF Vascular Endothelial Growth Factor
VHL Von Hippel-Lindau
XBP1 X-Box Binding Protein 1

1.INTRODUCTION

1.1 Background

1.1.1 Protein folding in the endoplasmic reticulum

The endoplasmic reticulum (ER) is an organelle that plays essential roles in multiple cellular processes that are required for normal and cellular survival functions. These processes include intracellular calcium homeostasis, protein folding and trafficking and lipid biosynthesis, (Anelli and Sitia, 2008), (Pizzo and Pozzan, 2007), Rizzuto et al., 2004).
The lumen of the ER constitutes an unique cellular environment. For instance, the ER contains the highest concentration of intracellular Ca^{2+} owing to active transport by Ca-ATPases. In addition, because of its role in protein folding and transport, the ER is rich in calcium-dependent molecular chaperones. ER-resident chaperones have multiple functions as they help folding nascent proteins, prevent unfolded proteins from aggregating, help protein refolding and thus rescue misfolded proteins, and sense the levels of accumulating misfolded proteins in the organelle. The ER lumen is also an oxidative environment relative to the cytosol, which is crucial for the formation of disulphide bonds in maturing proteins, further stabilizing the proteins structure. Moreover, various post translational modifications of proteins, including glycosylation and lipidation, occur in the ER and serves as signal of the protein's folding state. (Ma and Hendershot, 2004), (Schroder and Kaufman, 2005).
In eukaryotic cells, secreted and transmembrane proteins enter the ER as unfolded polypeptide chains; the flux of proteins entering the ER is variable because it can change rapidly in response to cell differentiation, to environmental conditions and according to the physiological state of the cell. Because many cell surface proteins relay important signals that ultimately determine cell fate, i.e whether a cell is to differentiate, divide, migrate, or die, the fidelity of folding and assembly of these components is vital for the health of an organism, as a result the ER has to constantly asses its folding capacity to meet the cellular needs.
An imbalance between the load of unfolded proteins that enter the ER and the capacity of the cellular machinery that handles this load is called ER stress. ER homeostasis can be perturbed by physiological processes such as aging, postprandial production of insulin and plasma cells differentiation, as well as by pathological processes such as viral infections, environmental toxins, inflammatory cytokines (Fig.1). ER stress is also triggered by conditions that alter proteostasis associated with perturbations in protein maturation, expression of certain mutant proteins, decreased chaperones function, abnormal ER calcium content, redox metabolism and altered protein trafficking (Kaufman, 2002; Kaufman et al., 2002).

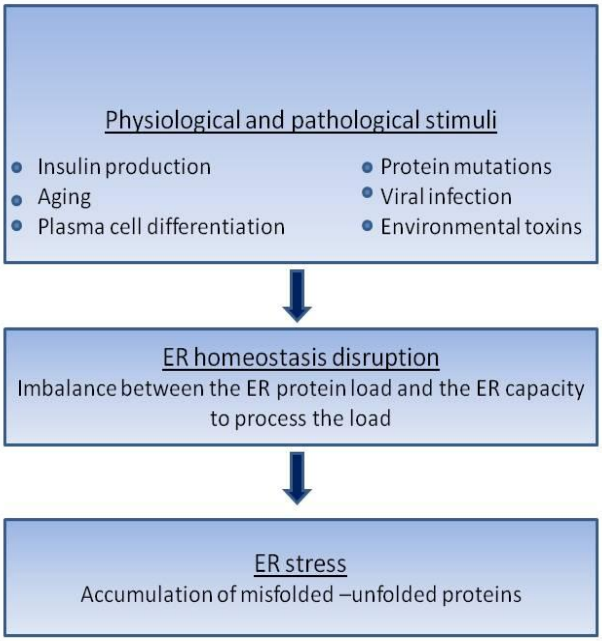

Fig. 1 The ER homeostasis. *The ER homeostasis can be perturbed by physiological and pathological stimuli. Disruption of ER homeostasis causes accumulation of unfolded and misfolded proteins in the ER. This condition is referred as ER stress.*

As an initial response to ER stress, cells activate the Unfolded Protein Response (UPR) to decrease the unfolded protein load and reestablish homeostasis. Gene expression microarray studies performed in *S. cerevisiae* showed that the UPR signaling enforces global changes in expression of molecules related to nearly every aspect of the secretory pathway, as activation of the UPR increases the amount of ER membrane and its components, including chaperones and protein folding enzymes (Cox et al., 1997).

In eukaryotes the UPR is initiated by three ER transmembrane proteins, inositol requiring 1 (IRE1), protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6). These master regulators sense the protein folding conditions and translate this information to the nucleus to regulate downstream effectors in order to attenuate stress and to restore homeostasis (Bernales et al., 2006) (Yoshida et al., 2003). These responses include: i) attenuation of protein translation to reduce ER workload and prevent further accumulation of unfolded proteins; ii) upregulation of molecular chaperones and processing enzymes to enhance the ER folding activity; iii) increased ER-associated degradation (ERAD) components to promote clearance of unfolded proteins by the proteasome

(Harding et al., 2000b), (Yan et al., 2002), (Yoshida et al., 2003), (Oyadomari et al., 2006).

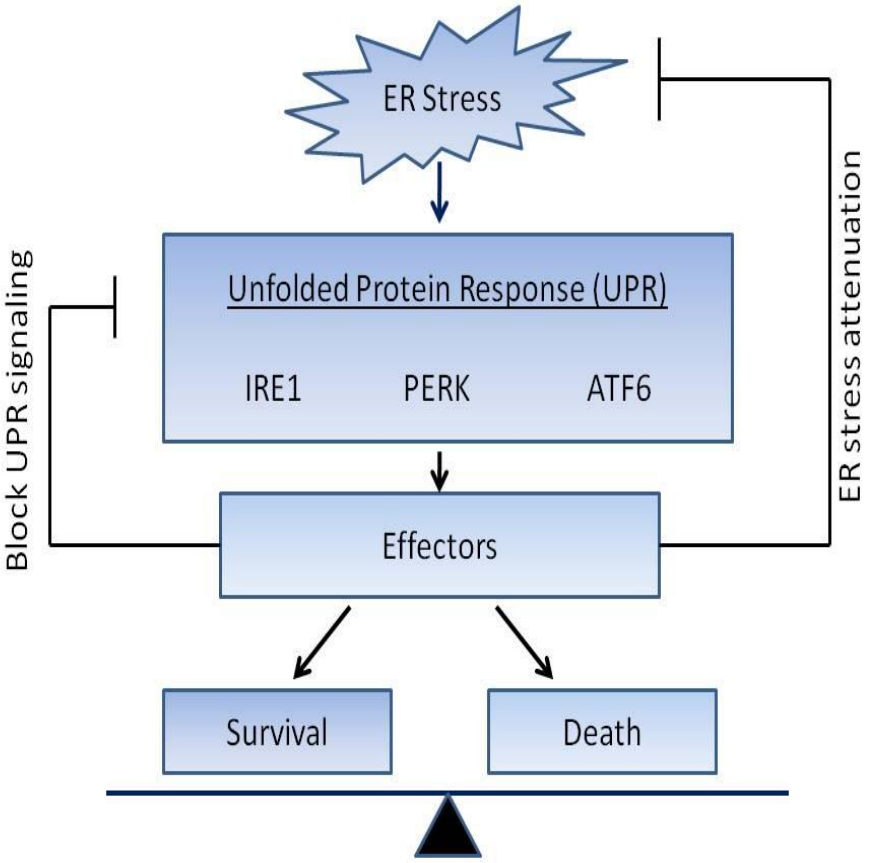

Fig. 2 The signaling network activated by the UPR*. The UPR is initiated by three master regulators IRE1, PERK and ATF6. Together these transducers regulate different effectors that attenuate ER stress and turn off the UPR when the ER homeostasis is restored, balance the survival/death signaling pathways.*

These signaling pathways are not unidirectional as the UPR is equipped with a set of effectors that function in negative feedback loops to provide tight control of the network, preventing harmful hyperactivation and terminating the signaling once homeostasis is restored (Fig.2).
Thus the UPR operates as a homeostatic control circuit that regulates the protein folding and secretion capacity of the cells according to need.

1.1.2 The Unfolding Protein Response signaling pathway

The UPR signaling is initiated by three ER transmembrane sensors, which have their luminal portions sensing the protein folding environment in the ER and the cytoplasmic effector portions that interact with the transcriptional or translational apparatus. The first stress transducer was identified in *S. cerevisiae* by screening for mutations that block the activation of a UPR-inducible reporter (Cox and Walter, 1996; Shamu et al., 1994). The gene in question encodes a type 1 ER –resident transmembrane protein with a novel luminal domain and a cytoplasmic portion containing a protein kinase domain (IRE1) (Cox et al., 1993). In response to the accumulation of unfolded proteins, IRE1 oligomerizes allowing for trans-autophosphorylation of juxtaposed kinase domains. To date it is still debated how oligomerization may occur. It has been hypothesized that oligomerization can be triggered directly by binding of unfolded proteins to the IRE1 luminal domain, or might involve the release of the oligomerization-repressing chaperone GRP78, or both (Credle et al., 2005) (Bertolotti et al., 2000; Hetz and Glimcher, 2009; Korennykh et al., 2009).
Activated IRE1 exhibits endoribonuclease activity and mediates the sequence specific cleavage of a single mRNA called X-Box-Binding Protein 1 (XBP1) in higher eukaryotes (Shamu et al., 1994), (Calfon et al., 2002). Spliced XBP1 (XBP1s) encodes a potent transcriptional activator, (Yoshida et al., 2001), while the unspliced XBP1 mRNA encodes XBP1u, an inhibitor of the UPR. IRE1 is therefore a bifunctional enzyme, possessing both a protein kinase and a site-specific endoribonuclease that is regulated by its intrinsic kinase module. In addition to post-transcriptional regulation by IRE1, XBP1 is also a transcriptional target of the UPR, in fact it has been shown that the levels of XBP1 mRNA increase upon UPR induction and continue to rise as ER stress declines and IRE1 is inactivated (Yoshida et al., 2006).

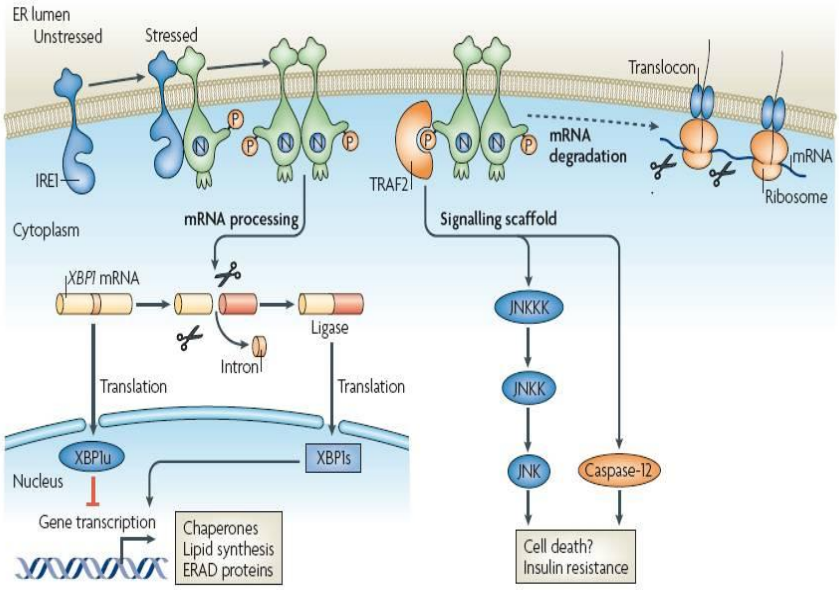

Fig.3 Signaling by IRE1 in stressed cells*. IRE1 oligomerizes in the plane of the ER membrane. Trans-autophosphorylation in its cytosolic kinase domain unmasks its endoribonuclease activity. IRE1 mediates sequence specific cleavage of the single known mRNA XBP1, and excises a small RNA intron fragment. The two ends of the mRNA are ligated, which leads to a frame shift in the coding sequence. Spliced XBP1 mRNA encodes a potent transcriptional activator (XBPs), whereas the unspliced XBP1mRNA encodes XBPu, an inhibitor of the UPR. In mammalian XBP1 upregulates a set of UPR genes that promote ER-associated degradation (ERAD) of misfolded proteins and ER biogenesis. IRE1 can also recruit TRAF2 to signal to JNK and alters intracellular signaling (for example, resulting in insulin resistance). The IRE1-TRAF2 complex has been linked to caspase-12 activation and cell death. In cultured Drosophila melanogaster cells, activated IRE1 can promote the cleavage of various ER-localized mRNAs, leading to their degradation, thus reducing the load on the stressed ER. From Ron, D. Nature Reviews 2007.*

The ER stress transducer protein kinase RNA (PKR)-like ER-kinase (PERK) evolved from IRE1 and their luminal stress-sensing domains are phylogenetically related and similar in structure and function (Bertolotti et al., 2000). The

cytoplasmic portion of PERK contains a protein kinase domain which undergoes activating trans-autophosphorylation by oligomerization in ER stressed cells. PERK phosphorylates the α-subunit of the eukaryotic translation initiation factor-2 (eiF2α) at Ser51; lower levels of eIF2α result in lower translation initiation, globally reducing the load of newly synthetized proteins which are destined to enter the already stressed ER lumen (Harding et al., 1999). Because other three eIF2 kinases, triggered either by amino acid starvation, double stranded RNA and heme depletion, can activate this pathway independently of ER stress, this portion of the UPR is termed the integrated stress response (ISR) (Harding et al., 2003) (Fig. 4).
Since eIF2α plays a key role in protein translation, cells must tightly regulate the level of phosphorylated eIF2α to survive. Two identified genes, GADD34 and CReP encode phosphatases that independently dephosphorylate eIF2α (Bertolotti and Ron, 2001; Bertolotti et al., 2000). CReP is constitutively expressed and contributes to the baseline eIF2α dephosphorylation, while GADD34 is induced as part of the gene expression program activated by eIF2α phosphorylation and serves as the negative feedback loop that operates within (Novoa et al., 2001).

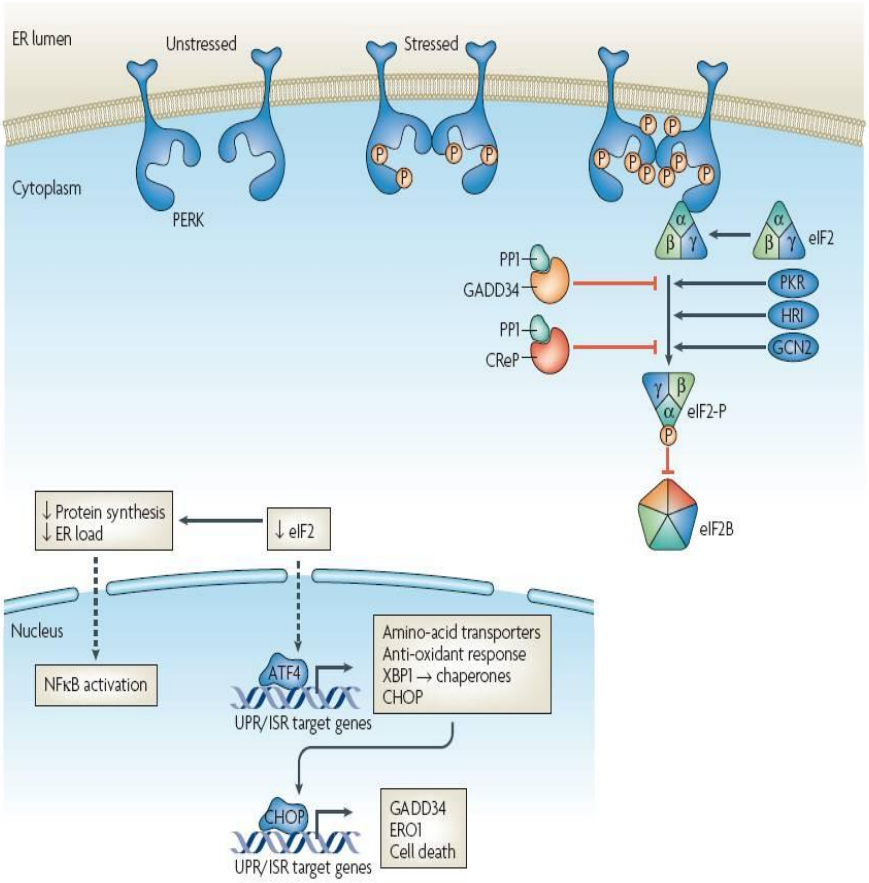

Fig. 4 Signaling by PERK to the translational machinery and the integrated stress response*. In response to ER stress PERK oligomerizes and is activated by trans-autophosphorylation of its activation loop. Phosphorylation of the single known substrate, the alpha subunit of eIF2 on Ser51 inhibits the pentameric guanine nucleotide exchange factor eIF2B from recycling eIF2 to its active GTP-bound form. Other eIF2 kinases (PKR, HRI and GCN2) can activate the pathway independently of ER stress, for this reason this portion of the UPR is termed the integrated stress response (ISR). Lower global protein synthesis reduces the ER unfolded protein load but also affect gene transcription. Translation of ATF4 is increased under conditions of limiting eIF2α, whereas NFkB is activated post-translationally. From Ron, D. Nature Reviews 2007.*

PERK activation not only promotes global translation attenuation but, paradoxically, enhances translation of a few set of mRNAs of which ATF4 is the

best studied member. The ATF4 mRNA contains multiple upstream open reading frames (uORF) that precede the ATF4 ORF. This molecular organization normally suppresses ATF4 protein synthesis when ribosomal assembly is efficient as ribosomes initiate translation at the uORF. However, during ER stress when ribosomal assembly are impaired, the uORFs are bypassed in favor of the ATF4 start codon (Harding et al., 2000b), (Harding et al., 2000a). ATF4 is a transcription factor and promotes expression of a set of genes that restore ER homeostasis.

ATF6 governs the third branch of the UPR and is the archetype of a growing family of transmembrane ER stress sensors, many of them expressed in a cell-type or tissue specific manner. ATF6 bears a ER luminal stress sensing domain coupled, via a transmembrane segment, to a cytosolic B-ZIP transcription factor domain (Adachi et al., 2008). In response to ER stress, ATF6 is transported to the Golgi where specific proteases cleave the transmebrane and liberate the cytosolic portion. The soluble cytosolic domain of ATF6 is a transcription factor that upon translocation to the nucleus upregulates transcription of a set of UPR target genes that overlap with those activated by XBP1 and ATF4 (Haze et al., 1999; Nadanaka et al., 2006; Yoshida et al., 2000). In this manner ATF6 is thought to protect the cell from ER stress (Fig. 5).

Recently CREBH, a bZIP-containing transcription factor, has been identified to mediate the acute-phase response in liver. CREBH is expressed mainly in hepatocytes , and its expression is induced by inflammatory cytokines, such as TNF-α , interleukin β and interleukin-6. When ER stress occurs, CREBH is activated and mediates the acute phase response in the liver. CREBH is activated through translocation from the ER to the Golgi apparatus where it is cleaved by S1P and S2P.the N-terminal fragment of CREBH translocates to the nucleus where it can induce transcription. However, CREBH does not induce the expression of genes involved in the UPR. Instead, it binds to a DNA-sequence motif in the promoter region of a subset of acute-phase-response genes, including the serum amyloid P component and C-reactive protein (Zhang and Kaufman, 2008).

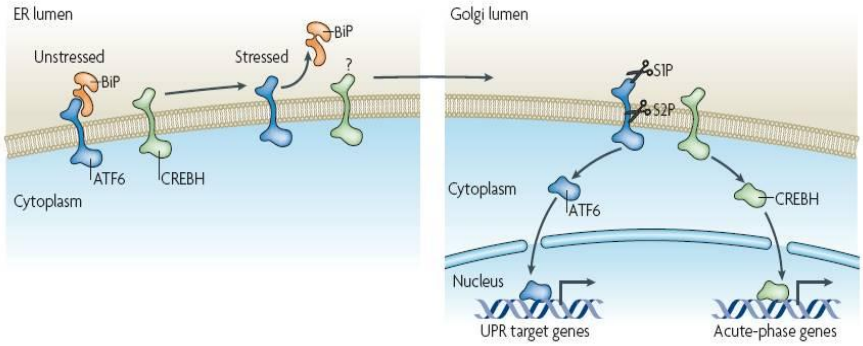

Fig.5 Signaling by ATF6*. ATF6 and CREBH are transmembrane proteins with a cytoplasmic portion that, when liberated from its transmembrane tether, can bind to DNA and activate genes. In unstressed cells ATF6 and CREBH reside in the ER membrane. ATF6 trafficking appears to be hindered by binding to the ER chaperone BiP to its luminal domain. ER stress disrupts BiP binding and ATF6 and CREBH are delivered to the Golgi apparatus. In the Golgi these proteins are subjected to two consecutive cleavage by S1P and S2P proteases which liberates the cytosolic portions of the proteins from the membrane and allows their import into the nucleus. ATF6 activates a set of UPR target genes not yet fully characterized, whereas CREBH activates the acute-phase response genes that encode secreted proteins involved in inflammation. From Ron, D. Nature Reviews 2007.*

1.1.3 Cross talk between the arms of the Unfolded Protein Response pathway

Whereas IRE1, ATF6 and PERK activation proceed independently in ER-stressed cells, the three arms of the UPR communicate with each other extensively. It has been found that the transcriptional effects of the three arms overlap significantly, which is achieved in part through mutual positive reinforcement.
Results from experiments performed with *C. elegans* indicate that there is a functional redundancy between the IRE1-XBP1 arm of the UPR and the ATF6 branch. This is also inferred from the experiments using knock out animals showing that while mutations on either arms of the UPR signaling are well tolerated, compromising both arms blocks worm development (Shen et al., 2001), (Shen et al., 2005). Furthermore, genes expression microarray analysis of mammalian cell lines show that XBP1 is transcriptionally activated by both ATF6 and PERK signaling (Yoshida et al., 2001), (Calfon et al., 2002) .
The transcription factor CHOP operates as a downstream component of the ER-stress, at the convergence of the IRE1, PERK, and ATF6 pathways. CHOP is a member of the C/EBP family of bZIP transcription factors that is induced by ER stress (Ma et al., 2002). The CHOP promoter contains binding sites for all of the major inducers of the UPR, including ATF4, ATF6, and XBP1 (Yamamoto et al., 2004) and different studies have provided evidence that these transcription factors have causative roles in inducing CHOP gene transcription.

1.1.4 Links between the Unfolded Protein Response and cell death

Although the UPR is designed to deal with the multitude of ER stresses in a timely and efficient manner in order to enhance cell survival, prolonged stress has severe consequences including apoptosis.
Depending on the particular state of the cells and what type of ER stress is encountered, the UPR can dynamically increase the folding capacity of the cells, and the ability to degrade misfolded unwanted proteins. However, if subjected to continuous stress, such that homeostasis is not regained, cells commit to apoptosis.
Increasing evidence indicates that the UPR regulates both apoptotic and survival effectors. The balance between the effectors depends on the nature of the ER stress, whether it is tolerable or irresolvable. It remains to be elucidated how the cell senses the level of stress and what is the nature of the switch. Up to now no trigger for ER stress has been identified that selectively elicits only protective response or only apoptosis. Instead ER stress activates all UPR signaling pathways, thereby simultaneously producing antagonistic outputs. It has been suggested that the duration of individual UPR branch activity may determine the cell outcome.
Moreover to date it is unknown how cells integrate the different pro-apoptotic signals to ultimately make a life/death decision. The choice to commit to cell death rather than display potentially malformed and malfunctioning protein receptors on the cell surface may be the ultimate solution to protect the organism from cells that

no longer respond to their environment, and hence may exhibit uncontrolled growth or differentiation.

The ER-proximal sensors PERK and IRE1 are able to activate both cell survival as well cell death pathways. While IRE1 leads to activation of the JNK and downstream caspases, PERK death-signaling pathway is mediated by the transcriptional activation of CHOP (Fig. 6).

IRE1 exhibits signaling functions beyond its nucleolitic activity. Recruitment of TRAF2 (Tumor necrosis factor receptor 2) allows the activation of the stress induced Jun N-terminal kinase (JNK) and the interaction with components of the cell death machinery, such as the ER-localized caspase 12 in mice (Urano et al., 2000), (Nishitoh et al., 2002; Nishitoh et al., 1998) and caspase 4 in human. The IRE1-JNK signaling pathway is also connected to the cell death mechanism by activation of the pro-apoptotic BCL-2 family member BIM, while inhibiting the anti-apoptotic protein BCL-2 (Hetz et al., 2006).

In addition it has been reported that IRE1 interacts with the BCL-2-associated X-protein (BAX) and BCL-2 antagonist/killer (BAK) therefore modulating the UPR signaling outcomes.

PERK signaling promotes cell death through specific transcriptional induction of the CHOP gene. Upregulated CHOP expression promotes cell death (McCullough et al., 2001), whereas CHOP deletion protects against the death of stressed cells (Oyadomari et al., 2002; Zinszner et al., 1998). These observations indicate the CHOP evolved to link insurmountable levels of ER stress to the cell-death machinery. However, alternative explanations for increased survival of ER stress CHOP $^{-/-}$ cells have emerged. It has been found that CHOP activates GADD34, reduced levels of GADD34 protein in CHOP$^{-/-}$ cells correlate with sustained elevation of phosphorylated eIF2α, sustained repression of translation, lower levels of unfolded ER proteins and, consequently, lower levels of ER stress (Marciniak et al., 2004). Therefore, the CHOP-dependent GADD34-mediated negative feedback on levels of phosphorylated eIF2α can, in some circumstances, be maladaptive because it encourages excessive recovery of the ER load in stressed cells.

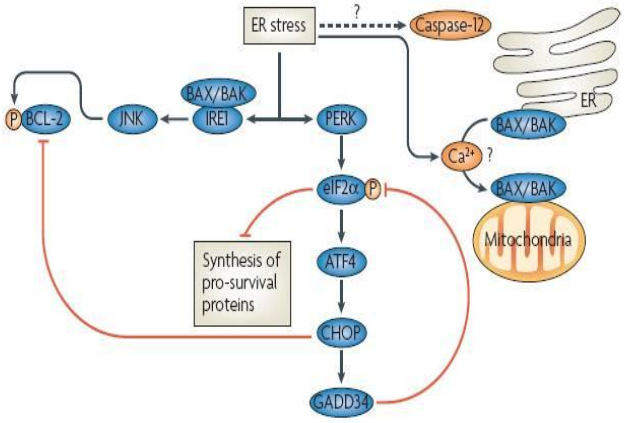

Fig.6 ER stress and cell death. Cell death by ER stress occurs by a porly understood mechanism. Altered Ca handling might be implicated in the translocation of the death effectors BAX and BAK from the ER to the mitochondria. In mice caspase 12 activation is implicated in cell death through TRAF2. IRE1-mediated activation of JNK contributes to cell death by phosphorylating and inactivating the anti-apoptotic regulator BCL2. The formation of a complex with the pro-death proteins BAX and BAK may assist in IRE1 activation. The transcription factor CHOP may repress BCL2 expression. CHOP is also engaged in a negative feedback loop that operates in the UPR to promote eIF2a dephosphorylation and recovery from protein synthesis. From Ron, D. Nature Reviews 2007.

1.2 Previous work

Secretory and transmembrane proteins that enter the ER have to fold in this compartment in order to reach their ultimate functional state. As polypeptides are folded in the ER, sugars are added and disulfide bonds are formed. All of these essential processes are helped by ER-resident proteins, such as molecular chaperones and protein modifying enzymes. Physiologic stresses, such as increased secretory load, change in oxygen availability or pathological stresses, such as the presence of mutated proteins that cannot properly fold in the ER, can lead to an imbalance between the demand for protein folding and the capacity of the ER for proper folding. When this imbalance is detected a signal is sent to the nucleus where numerous appropriate genes are switched on to produce more ER and to help protein folding. In this way, the UPR allows the cell to adjust the amount of organelle and ER components, according to need. It is one of the many homeostatic mechanisms by which cells keep their various constituent parts in proper balance.

In **chapters 1.2.1.1; 1.2.1.2 and 1.2.1.3** we analyzed the folding requirements for efficient secretion of coagulation factor VIII (FVIII) a large glycoprotein that is deficient in the X chromosome-linked bleeding disorder hemophilia A. As FVIII is prone to misfolding in the ER lumen, its expression provides a unique approach to study and manipulate the ER stress response that does not rely on pharmacological intervention.

Our next step was to assess whether different cells and tissues may be able to modulate different components of the complex UPR pathway in response to specific cellular functions or environmental conditions. Our experimental work used as a biological model the plasma cells, the Ig-secreting effectors of the immune system, as they represent an ideal model to investigate the cellular dynamics of proteosynthetic stress. Moreover, it has been reported that during plasma cell differentiation ER-resident proteins are synthesized before Ig accumulation. These observations suggest that the activation of certain UPR elements may be driven by specific developmental program(s), rather than being solely a consequence of cargo accumulation.

In **chapters 1.2.2.1 and 1.2.2.2** we reported that the proteasome capacity dramatically decreases along with plasma cell differentiation. In striking correlation with impaired proteolysis, polyubiquitinated proteins accumulate, death-inducing proteins are stabilized, presumably via competitive inhibition of their degradation, and apoptotic sensitivity to proteasome inhibitors ensues, prior to spontaneous apoptosis thus attesting to a cause–effect relationship between proteasomal overload and death.

1.2.1 Posttranslational requirements for Factor VIII folding result in the endoplasmic reticulum stress

Factor VIII (FVIII) is the X-chromosome –linked gene product that is defective in the bleeding disorder hemophilia A. FVIII functions in the blood-clotting cascade as the cofactor for FIXa proteolitic activation of FX.To date there are no known primary or natural established cell lines that express FVIII, thus our knowledge of FVIII synthesis and secretion is derived from interpretation of results obtained by transfection of mammalian cells with plasmid vectors containing the FVIII cDNA. FVIII expression in these systems is two to three orders of magnitude lower than that observed with other genes, including the homologous Factor V, using similar expression strategies.

The biosynthesis, folding and secretion of FVIII were studied to elucidate the mechanisms responsible for the low level of FVIII expression in transfected mammalian cells. At least three mechanisms were identified that limit FVIII expression: 1) FVIII mRNA is inefficiently expressed, 2) high levels of von Willebrand Factor are required to promote stable accumulation of FVIII, 3) the primary translation product is inefficiently folded and transported from the endoplasmic reticulum (ER) to the Golgi apparatus.

The work performed in Kaufman laboratory at HHMI focused mainly in two area of research:

i) Identification of the structural requirements for efficient FVIII protein synthesis, assembly and secretion
ii) Identification of the intracellular factors that limit FVIII secretion

i) FVIII is synthesized as a single polypeptide chain of 2351 amino acids from which the first 19 residues, corresponding to a hydrophobic signal peptide, are removed in the ER. Internal sequence homologies of the protein indicate a triplicated A domains homologous to the A domains of factor V and ceruloplasmin, a duplicated C domain and a large B domain that is not homologous to any other known structure and that is dispensable for FVIII activity. Several N-linked glycosylation sites, are modified in the endoplasmic reticulum (ER). Upon secretion from the cells FVIII is further processed by cleavage to yield a heterodimer consisting of a 220 kDa amino-terminal heavy chain, associated in a metal ion-dependent manner with a carboxyl-terminal-derived light chain.

The evolutionary and structural conservation between the coagulation factors and the blue oxidases suggests a role for copper ion binding in the coagulation factors. We have studied the copper ion oxidation state and the ligands that coordinate copper ion binding in FVIII by atomic absorption spectroscopy, site-directed mutagenesis and expression of FVIII cDNA. The results evidence that a single molecule of reduced copper Cu(I) is buried within FVIII and is released and oxidized to Cu(II) upon treatment with EDTA. Site-directed mutagenesis and functional characterization of the expressed mutated proteins allowed the identification of the Cys 310 as the ligand within the type-1 copper binding site.

ii) During its folding in the ER, FVIII binds to the chaperone BiP and to the lectin proteins calnexin and calreticulin. Binding to BiP correlates with its inefficient secretion. Altogether these three ER resident chaperones exert a tight “quality control’ on FVIII folding and have a negative effect on the secretion process causing ER-stress. In addition FVIII requires ATP for release from BiP and

secretion. We demonstrated that FVIII forms transient aggregates immediately after its synthesis in the ER. We have studied the properties of this aggregation to conclude that aggregate formation increases with the FVIII expression level and is reversible. The aggregates do not contain interchain disulfide bonds, although they do require ATP for disaggregation. A hydrophobic region within FVIII, between amino acid residues 227-336, was identified that may predispose FVIII to aggregation. When this region was replaced by the homologous residues from FV, the extent of aggregation was significantly reduced.

1.2.1.1 Identification and Functional Requirement of Cu(I) and Its Ligands within Coagulation Factor VIII

ABSTRACT: Coagulation factor VIII (FVIII) is a heterodimer consisting of a light chain of 80 kDa (domains A3-C1-C2) in a metal ion-dependent association with a 220-kDa heavy chain (domains A1-A2-B). The nature of the metal iondependent association between the heavy and light chains was investigated using atomic absorption spectroscopy, electron paramagnetic resonance spectroscopy (EPR), and site-directed mutagenesis and expression of the FVIII cDNA. Whereas copper ion was not detected in intact recombinant FVIII, EDTA dissociation of the chains yielded an EPR signal consistent with 1 mol of Cu(I)/mol of active protein, supporting the hypothesis that a single molecule of reduced copper ion is buried within intact FVIII and is released and oxidized upon treatment with EDTA. Cu(I), and not Cu(II), was able to reconstitute FVIII activity from dissociated chains, demonstrating a requirement for Cu(I) in FVIII function. Three potential copper ion binding sites exist within FVIII: one type-2 site and two type-1 sites. The importance of these potential copper ion ligands was tested by studying the effect of site-directed mutants. Of the two histidines that compose the type-2 binding site, the His-19573Ala mutant displayed secretion, light and
heavy chain assembly, and activity similar to wild-type FVIII, while mutant His-993Ala was partially defective for secretion and had low levels of heavy and light chain association and activity. In contrast, FVIII having the mutation Cys-310 3 Ser within the type-1 copper binding site in the A1 domain was inactive and partially defective for secretion from the cell, and the heavy and light chains of the secreted protein were not associated. Mutant Cys-2000 3 Ser within the A3 domain displayed secretion, assembly, and activity similar to that for wildtype FVIII. These results support the hypothesis that Cu(I) is buried within the type-1 copper binding site within the A1 domain and is required for FVIII chain association and activity.

1.2.1.2 ATP-Dependent Dissociation of Non-Disulfide-Linked Aggregates of Coagulation Factor VIII Is a Rate-Limiting Step for Secretion

ABSTRACT: Deficiency in coagulation factor VIII leads to the bleeding disorder hemophilia A. Previous studies demonstrated that factor VIII secretion is limited due to an ATP-requiring step early in the secretory pathway. In this report, we identified that this ATP-dependent rate-limiting step involves the dissociation of non-disulfide-linked aggregates within the endoplasmic reticulum (ER). In contrast to the numerous examples of interchain disulfide-linked aggregates, factor VIII is

the first protein characterized to form non-disulfide-linked high molecular weight aggregates within the ER. Approximately a third of newly synthesized factor VIII was detected in high molecular weight aggregates. These aggregates disappeared over time as functional factor VIII appeared in the medium. The aggregated complexes did not require proteasomal degradation for clearance. Aggregate formation was enhanced by ATP depletion, and upon restoration of metabolic energy, these aggregates were dissociated and secreted. With the coexpression of von Willebrand factor (vWF), a small portion of vWF coaggregated with factor VIII. However, vWF dissociated from the aggregates more rapidly than factor VIII, supporting that these aggregates are dynamic. An increase in the factor VIII expression level elicited a corresponding increase in the fraction of factor VIII that was aggregated. In addition, a 110 amino acid sequence containing a hydrophobic *â*-sheet within factor VIII was identified that may predispose factor VIII to aggregation. These data show that formation and ATP-dependent dissolution of nondisulfide-linked factor VIII aggregates is a dynamic, rate-limiting step during the folding process in the early secretory pathway. In summary, we have identified an unprecedented requirement for protein transport out of the ER that involves an ATP-dependent dissociation of non-disulfide-linked aggregates within the ER.

1.2.1.3. Biosynthesis, assembly and secretion of coagulation FVIII

Factor VIII is a large complex glycoprotein that is deficient in hemophilia A. It has a domain organization consisting of A1-A2-B-A3-C1-C2 where the B domain is a heavily glycosylated region that is dispensable for procoagulant activity. Factor VIII expression is a 10- to 20 fold lower than the homologous factor V. Factor VIII expression is limited due to a low level of steady-state messenger RNA in the cytoplasm and inefficient transport of the primary translation product from the endoplasmic reticulum to the Golgi apparatus. Within the secretory pathway, factor VIII is processed to a heterodimer of a heavy chain (domains A1-A2-B) in a metal ion association with the light chain (domains A3-C1-C2). Upon secretion from the cell, von Willebrand factor binds the light chain of factor VIII and stabilizes the factor, preventing degradation. Protein folding within the mammalian secretory pathway is facilitated by molecular chaperones. Within the endoplasmic reticulum, factor VIII exhibits stable interaction with protein chaperones identified as the immunoglobulin-binding protein (BiP), calnexin and calreticulin. BiP is a peptide dependent ATPase that interacts with exposed hydrophobic surfaces on unfolded proteins or unassembled protein subunits. A potential BiP binding site within factor VIII has been identified. Mutation of a single amino acid residue in the potential BiP binding site increased the secretion efficiency of factor VIII by threefold. Interestingly, the proposed BiP binding site is adjacent to a type-1 copper binding site within the A1 domain that is required for interaction between the factor VIII A1 domain and the A3 domain. We propose that Cu(I) binds the type-1 copper ion-binding site in the A1 domain and provides the essential requirement for a stable interaction between the heavy and light chains. Calnexin and Calreticulin are transmembrane and luminal proteins, respectively, localized to the endoplasmid reticulum, which associate transiently with many soluble and membrane glycoproteins during folding and subunit assembly. The calnexin and calreticulin interaction with factor VIII occurs primarily through amino-terminal linked

oligosaccharides within the heavily glycosylated factor VIII B domain and this interaction appears to be required for factor VIII secretion. The findings suggest that factor VIII cycles through interactions with BiP, calnexin and calreticulin. Although the interaction with BiP does not appear to be required for factor VIII secretion, data suggest that the calnexin and/or calreticulin interaction is required for secretion. The observations suggest a unique requirement for carbohydrate processing and calnexin/calreticulin interaction that may limit the productive secretion of factor VIII and have implications for approaches towards somatic cell gene therapy for hemophilia A.

1.2.1.4 Concluding remarks

The studies performed in Kaufman' lab used an unique powerful protein model system to elucidate the functional roles of carbohydrate processing, chaperones interaction and ER-stress that limit the productive secretion of FVIII and may have implications for approaches towards somatic cell gene therapy for hemophilia. Most importantly, these studies highlighted how inefficient FVIII secretion correlates with Interaction with the protein chaperone identified as the immunoglobulin binding protein (BiP), also called glucose-regulated protein of 78 kDa (GRP78) within the lumen of the ER. This chaperone is expressed in all cell types and is a member of the heat-shock protein family whose expression is induced at the transcriptional level by the presence of unfolded proteins or unassembled protein subunits within the ER.
BiP interaction with unfolded proteins or protein folding intermediates is a common feature of many secretory and transmembrane proteins that could not negotiate the stringent "quality control" system within the ER. This process involves a variety of mechanisms that collectively ensure that only correctly folded, assembled and modified proteins are transported along the secretory pathway. In contrast, non-native proteins are retained and eventually targeted for degradation.

1.2.2 The physiological role of the unfolded protein response: shaping B lymphocytes differentiation and limiting plasma cells lifespan

The occurrence of ER stress is observed in many physiological processes, especially in highly secretory cells such as plasma B lymphocytes, salivary gland and pancreatic β cells. The high demand for efficient protein folding and secretion processes in these cells constitutes a constant source of stress initiated by the presence of large amount of misfolded proteins that are normally generated during protein maturation. These folding sub-products are eliminated through ER-associated degradation (ERAD), where misfolded proteins translocate to the cytosol and are degraded by the proteasome.
To explore the ER stress in cells adaptation and death in physiological process we used two cell models as prototypes of professional secretory cells: i) antibody-secreting plasma cells derived from mice spleen and ii) B cell lymphoma that can be induced to differentiate in vitro. Plasma cells are the end-stage effectors of the humoral immune response, producing large amount of secretory immunoglobulins (IgM), being capable to release thousands Ig molecules per seconds. To achieve

these standards they expand the organelles devoted to protein synthesis, transport and secretion, and in particular the endoplasmic reticulum (ER) where Igs are synthesized, folded and assembled. How is this metamorphosis coordinated? Proteomic analysis of B cells induced to differentiate with lipopolysaccharide (LPS) shows that the LPS-stimulated B cells activate a developmental program that induces the stepwise synthesis of different class of proteins so as to increase the secretory capacity. After LPS induction ER-resident proteins increase over the entire time whereas IgM subunits increase significantly only after 2-3 days of LPS treatment, to become the dominant molecular species synthesized in the last days of differentiation.
LPS-stimulated B cells begin to die after day 3 or 4, concomitantly with massive IgM secretion and accumulation of spliced XBP1. This finding, together with previous observation that XBP1 is essential for plasma cell development, suggested that exuberant Ig synthesis causes stress, induces ER biogenesis and eventually apoptosis via UPR-related pathways.
While confirming a correlation between massive Ig production and plasma cell death, our finding reveal that the proteasomal capacity decreases during plasma cell differentiation, despite the higher demand imposed by the high rates of proteins production. Thus an unfavorable proteasomal load versus capacity can predispose cells to apoptosis. This finding is further supported from the fact that proteasome inhibitors have been proved particularly effective in the therapy of multiple myeloma by inducing apoptosis of malignant cells.

1.2.2.1 The making of a professional secretory cell: architectural and functional changes in the ER during B Lymphocyte plasma cell differentiation

ABSTRACT: B lymphocytes are small cells that express antigen receptors and secrete little if any IgM. Upon encounter with antigen, they differentiate into short-lived plasma cells, which secrete large amounts of polymeric IgM. Plasma cell differentiation entails a massive development of the endoplasmic reticulum to sustain high levels of Ig production. Recent findings suggest a role for the unfolded protein response in orchestrating the architectural and functional changes during terminal plasma cell differentiation.

1.2.2.2. Progressively impaired proteasomal capacity during terminal plasma cell differentiation

ABSTACT: After few days of intense immunoglobulin (Ig) secretion, most plasma cells undergo apoptosis, thus ending the humoral immune response. We asked whether intrinsic factors link plasma cell lifespan to Ig secretion. Here we show that in the late phases of plasmacytic differentiation, when antibody production becomes maximal, proteasomal activity decreases. The excessive load for the reduced proteolytic capacity correlates with accumulation of polyubiquitinated proteins, stabilization of endogenous proteasomal substrates (including Xbp1s, and Bax), onset of apoptosis, and sensitization to proteasome inhibitors (PI). These events can be reproduced by expressing Ig-l chain in nonlymphoid cells.

Our results suggest that a developmental program links plasma cell death to protein production, and help explaining the peculiar sensitivity of normal and malignant plasma cells to PI.

1.2.2.3 Concluding remarks

Physiologically the UPR is thought to monitor the demand placed on the ER folding machinery and allow cells to promptly adapt to novel developmental requests. Therefore the remarkable increase in Ig production that accompanies B to plasma cell differentiation could drive the expansion of the ER via the UPR. Of note, XBP1$^{-/-}$ mice lacks plasma cells and serum Igs, despite having normal numbers of B lymphocytes. It is thus clear that XBP1 is essential for terminal B cell differentiation.

The extensive similarity between the luminal domains of IRE and PERK create a paradoxical situation for plasma cells. On one hand, these cells require XBP1s and hence an active IRE1 pathway. On the other hand PERK mediated translational attenuation would be detrimental for the plasma cells mission, that is, massive antibody production. Recent experiments shows that B lymphocytes are capable of activating all three branches of the UPR pathway if challenged with pharmacological stressors like tunicamycin or thapsigargin. However, in response to plasma cells differentiation signals, a modified UPR is initiated. IRE1 is activated early, leading to XBP1 splicing. Perk shows sign of partial activation with kinetic similar to IRE1, but this is not sufficient to phosphorilate the eIF2α or to induce its downstream targets. These events occur prior to the upregulation of Ig transcripts or proteins, arguing that this is not the signal for their activation. ATF6 appears to be activated considerably later and could be activated by increasing processing of Ig proteins (Ma et al., 2010).

Our results show that the proteasomal capacity decreases in differentiating plasma cells, despite the higher demand imposed by high rates of Igs production. The stabilization of two pro apoptotic proteasome substrates known to control plasma cells lifespan as Bax and Bim may, at least in part, explain their relative increase with respect with Bcl2, until the death threshold is reached. In this scenario plasma cell death would be linked to Ig production, thus contributing to end humoral response.

The finding that an unfavorable proteasomal load/capacity ratio can predispose cells to apoptosis has important implications for the physiology of the immune system and for Ig-secreting tumors. In fact, in myeloma the reliance on the UPR is not diminished by neoplastic transformation and it may provide a method for selectively targeting myeloma cells, and potentially other cancers, which are known to be secretory and therefore dependent on the UPR.

1. 3 Cell stress and hypoxia

1.3.1 The hypoxia signaling pathway

Access to oxygen is crucial for most organisms, even small fluctuations in oxygen levels pose a threat and molecular mechanisms have evolved in multicellular organisms to sense and adapt to changes in oxygen levels. When oxygen becomes limiting, cells respond by resetting their cellular metabolism to a low oxygen consumption mode and minimizing the influence of free oxygen radicals. Cells decrease the oxidative phosphorylation and rely on glycolysis as the primary means of ATP production by upregulating the expression of genes that encode glycolitic enzymes and glucose transporters. At the tissue level the adaptation to low oxygen regulates several physiological parameters to increase the oxygen supply. Therefore, hypoxia enhances vascularization and de novo angiogenesis by upregulation of the angiogenic factor vascular endothelial factor A (VEGFA) and improves the oxygen carrying capacity of blood by enhancing the expression of erythropoietin (EPO).

In mammalian cells the hypoxia-induced transcription factor (HIF)1α is a master regulator of O_2 homeostasis. HIF-target genes enable cells to survive oxygen deprivation by providing oxygen-independent means of ATP production and/or by inhibiting hypoxia-induced apoptosis.

HIFs facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating the expression of genes that are involved in many cellular processes, including glucose uptake and metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis. HIF proteins are member of the PAS (PER-ARNT arylhydrocarbon receptor nuclear translocator-SIM) family of basic transcription factors that bind to DNA as heterodimers composed of an oxygen sensitive alpha subunit and a constitutively expressed beta subunit. To date three HIFs (HIF 1, 2, 3) have been identified, that regulate transcriptional programs in response to low oxygen levels. While HIF1 is ubiquitously expressed, HIF2 is only expressed in endothelial cells and in the kidney, heart, lungs and small intestine. HIF3 is expressed in the thymus, cerebellar Purkinje cells and the corneal epithelium of the eye (Gordan and Simon, 2007).

HIF1 is a heterodimeric protein that is composed of a constitutively expressed HIF1β subunit and an O_2-regulated HIF1α subunit. HIF1α is subjected to O_2-dependent hydroxylation on proline residue 402 and/ or 564 by prolyl hydroxylase domain proteins (PHDs) and this modification creates an interface for interaction with the von Hippel-Lindau tumor suppressor protein (VHL) (Fig.8), which recruits an E3 ubiquitin-protein ligase that catalyzes polyubiquitination of HIF1α, thereby targeting it for proteasomal degradation (Brahimi-Horn et al., 2007; Lum et al., 2007). Hence, HIF activity is regulated by a cycle of synthesis and oxygen-dependent degradation under normoxic conditions. Under hypoxic conditions, decreased molecular oxygen diminishes the activity of PHDs and these are further inhibited by reactive oxygen species (ROS) released from inefficiently respiring mitochondria (Brunelle et al., 2005; Guzy et al., 2005; Kaelin, 2005; Lu et al., 2005). The ROS oxidize and inactivate the ferrous ion within the active sites of the

PDHs. This renders the PDHs incapable of modifying the HIF1α subunit, resulting in the stabilization of HIFs. The stabilized HIF1α, dimerizes with HIF1β, binds to a core DNA binding sequence 5'-RCGTG-3' (R, purine A or G) in target genes, recruits the co-activators CREB and p300, and activates transcription (Jiang et al., 1996) (Mahon et al., 2001)(Fig.8). HIF1 heterodimers transactivate genes involved in pH regulation, glucose transport, glycolysis and vasculogenesis, allowing for cellular adaptation to hypoxia (Gordan and Simon, 2007; Semenza, 2003). HIF2α is a protein with extensive sequence homology to HIF1α that is also regulated by proline and asparagines hydroxylation, dimerizes with HIF1β and activates transcription of a group of target genes that overlap with, but is distinct from, those regulated by HIF1α (Jain et al., 1998), (Tian et al., 1997), (Wiesener et al., 2003) (Fig.7). HIF3α is an inhibitor of HIF1 that may be involved in feedback regulation because its expression is transcriptionally regulated by HIF1(Gu et al., 1998), (Makino et al., 2001).

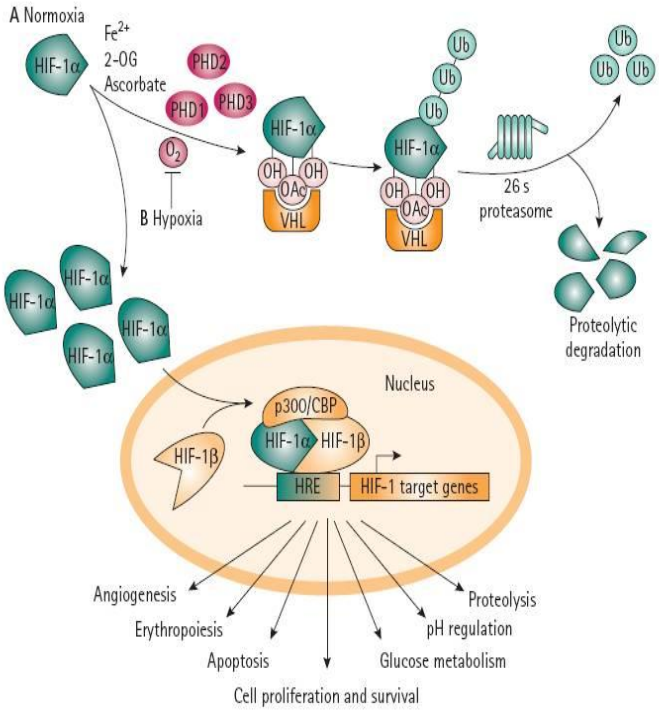

Figure 7. HIF regulation by proline hydroxylation. *(A) In normoxia, HIF1α is hydroxylated by proline hydroxylases (PHD1,2,3) in the presence of oxygen, Fe^{2+}, 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF1α(OH) is recognized by*

the product of the von Hippel-Lindau tumor suppressor gene pVHL, which, together with a multisubunit ubiquitin ligase complex, tags HIF1α with ubiquitin. This allows recognition by the proteasome and subsequent degradation. Acetylation of HIF1α(OAc) also promotes pVHL binding. (B) In response to hypoxia, proline hydroxylation is inhibited, VHL is not longer able to bind and target HIF1α for proteasomal degradation, which leads to HIF1α accumulation and translocation to the nucleus. HIF1α dimerizes with HIF1β, binds to the hypoxia response elements (HREs) within the promoters of target genes and recruits transcriptional coactivators such as p300/CBP for full transcriptional activity. A range of cellular functions are regulated by the target genes. From Stewart et al. BJUI, 2009.

However, HIF-1α activation alone may not account for the full repertoire of changes that occur when O_2 decreases and other hypoxia-dependent paths are activated independently of HIF-1α.
More recently, two other pathways that independently influence gene expression and processes of importance for cell growth have proved to be O_2-sensitive. The first occurs through regulation of an important integrator of metabolic signals, the kinase mammalian target of rapamacyn (mTOR, also known as FRAP1) and its downstream effectors that orchestrate the initiation of protein synthesis, autophagy and apoptosis sensitivity (Arsham et al., 2003). The second occurs through activation of the UPR. Hypoxia has been demonstrated to influence several components of both of these pathways, each of which is uniquely dependent upon the severity and/or duration of the hypoxic stress. Consequently, the cellular response to hypoxia may demonstrate a significant heterogeneity (Fig. 8).

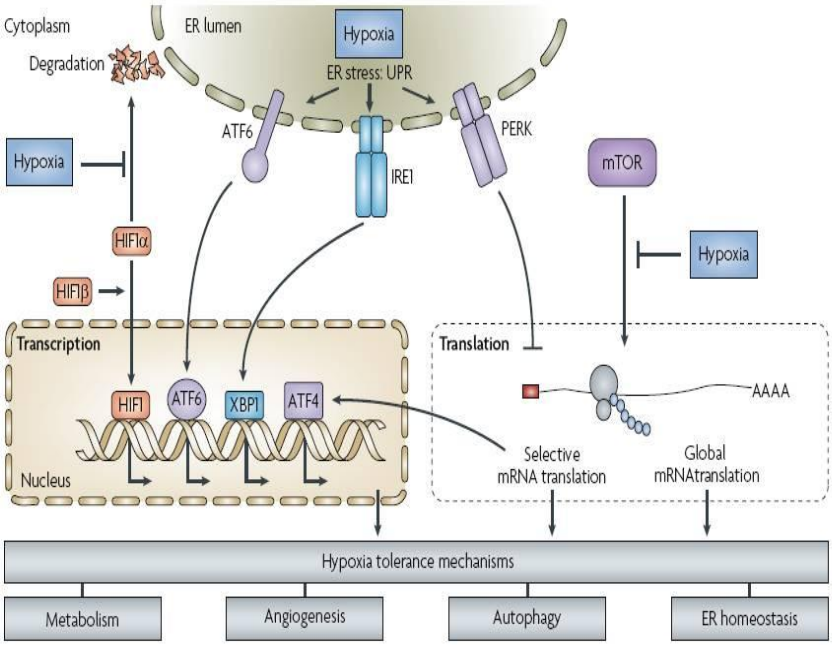

Figure 8. Cellular oxygen-sensing pathways*. Three main O_2-sensing pathways promote hypoxia tolerance by regulating the mRNAs transcription and translation. First hypoxia stabilizes the HIF1α facilitating its heterodimerization with HIF1β and transcriptional activation of downstream genes. Second, hypoxia triggers the UPR by activation of the ER-stress sensors PERK, IRE1 and most probably ATF6. IRE1 and ATF6 both induce a transcriptional response, while PERK causes inhibition of translation. Third, the activity of mTORC1, a complex containing the mammalian target of rapamycin (mTOR) kinase, which integrates and transmits positive and negative growth signals to the translational machinery, is inhibited by hypoxia. Changes in mRNA translation can be highly gene-specific and thus can also significantly contribute to differential protein expression during hypoxia. Together these three pathways influence the phenotype of hypoxic cells by altering metabolism, angiogenesis, autophagy and ER homeostasis. From Wouters et al. Nature Reviews Cancer, 2008.*

1.3.2 Unfolded Protein Response activation during hypoxic stress

Hypoxic activation of ER stress sensors strongly suggests that the lack of O_2 perturbs ER homeostasis. However, the direct requirement for O_2 in ER function remains to be fully clarified. The immediate phosphorylation of eIF2α upon anoxic exposure suggests a direct role for O_2 in ER protein maturation, involving several steps of oligosaccharide modifications, disulphide bonds formation, isomerization, quality control and export. Any of these processes may harbor requirements for O_2. In vitro, O_2 can supply the oxidative potential to drive disulphide bonds formation of a client protein by FAD, ER-oxidase 1 (ERO1) and Protein Disulphil Isomerase (PDI) (Tu and Weissman, 2002). It remains to be established whether O_2 is a crucial electron acceptor in the ER of mammalian cells in vivo, and whether this can account for the limited ER function during hypoxia

Little is known about the hypoxia associated changes involving PERK, IRE1 and ATF6 that affect the sensitivity to UPR activation. However, the cellular consequences of their activation are mediated by four distinct pathways that collectively act to alleviate ER stress and diminish its toxic effects. Each of these effectors pathways can influence the phenotype of hypoxic cells in diverse ways. The effector pathways include two “preventative” pathways ,that mediate inhibition of mRNA translation and induction of protein maturation machinery. Together these pathways function to prevent further accumulation of unfolded protein in the ER (Koritzinsky et al., 2006). At the same time UPR dependent processes stimulate ER-associated degradation (ERAD) and autophagy to remove potentially toxic misfolded proteins or protein aggregates that may have already accumulated (Fig 9).

Regulation of gene-specific mRNA translation. Inhibition of overall protein synthesis has long been known to be a hallmark of hypoxia. Part of this inhibition is mediated through regulation of the eukaryotic Initiation factor 4 (EIF4) through mTOR-influenced pathway. However, the majority of this effect during acute exposures to severe hypoxia is due to PERK-dependent phosphorylation of eIF2α (Koritzinsky et al., 2006), (Koritzinsky et al., 2007; Koumenis et al., 2002). As is the case for mTOR, inhibition of translation through PERK-eIF2α is expected to affect both energy homeostasis and specific gene expression. The transcript best characterized in this respect is ATF4, the 5’ UTR of which contains two conserved upstream open reading frames (uORFs) that prevent efficient translation under normal conditions. Reduction of the availability of non-phosphorylated eIF2α reduces initiation at the second uORF and increases the chance that initiation occurs at the *bona fide* start codon (Lu et al., 2004; Vattem and Wek, 2004). Consequently, ATF4 is induced during acute hypoxia in a PERK and eIF2α-phosphorylation-dependent manner (Koritzinsky et al., 2006), (Blais et al., 2004) . ATF4 is an important transcription factor and has been shown to be overexpressed in cancer primarily in hypoxic, perinecrotic regions of human tumors (Ameri et al., 2004). Selective synthesis of ATF4 during hypoxia also appears to be required for maintaining hypoxia tolerance (Bi et al., 2005). Several other transcripts of relevance to hypoxia are also selectively translated in a PERK-dependent manner including VCIP (VEGF and type 1 collagen-inducible protein) and matrix metalloproteinase 13 (MMP13). These effects may explain the defects in

angiogenesis that contribute to the poor growth of PERK-deficient tumors (Blais et al., 2006).

The transient inhibition of mRNA translation can also liberate the translational machinery (eukaryotic initiation factors, ribosomes) from pre-existing translation competent transcripts, such that newly made mRNAs may be able to more effectively compete for the translational machinery. This shift in translation has been referred to as "translational reprogramming, (Ron and Walter, 2007) and may allow for a bias in the synthesis of hypoxia-inducible mRNAs.

Regulation of protein maturation machinery. Many proteins involved in ER-localized protein folding and maturation have been reported to be upregulated during hypoxic conditions. These includes chaperones such as BiP and GRP94 (Murphy et al., 1991), (Roll et al., 1991), (Wilson and Sutherland, 1989) as well as oxidoreductases such as ERO1L (Romero-Ramirez et al., 2004), (Gess et al., 2003), and PDI (Tanaka et al., 2000). Although their induction is predicted to improve ER function during hypoxia and potentially mitigate the toxicity associated with accumulation of protein aggregates, this has not yet been formally shown. UPR-dependent upregulation of the protein maturation machinery in the ER might also contribute to increased secretion of angiogenic factors. Blockade of the UPR through expression of a dominant-negative IRE1 has been shown to reduce VEGFA secretion and result in smaller tumors with reduced vascular density. Similarly, knockdown of ERO1L decreased the ratio of extracellular VEGFA protein to intracellular mRNA levels, at least under normoxic conditions (May et al., 2005).

Regulation of ER-associated degradation. There is a strict quality control of protein folding in the ER, which is applied before the proteins exit to the Golgi. If the battery of maturation proteins fails to produce a *bona fide* folded protein, the aberrant protein can be targeted for ERAD through mannose trimming of N-linked glycans. ERAD is an important detoxification mechanism that prevents protein aggregation and cytotoxycity. The importance of ERAD in mediating hypoxia tolerance has yet to be investigated, but several proteins involved in ERAD are induced by hypoxia. These include HERP, suggesting a link between client proteins and the proteasome, SEC61A1 (translocon subunit) (Romero-Ramirez et al., 2004) and synoviolin (a ubiquitin ligase) (Qi et al., 2004) and Derlin 3 (our results).

Regulation of autophagy. Growing evidence suggests that autophagy is also influenced by the UPR in response to ER stress. In some cases autophagy appears to be mediated by PERK, whereas in others it occurs downstream of IRE1. For example, aggregation of polyglutamine polypeptides, associated with Huntington disease, induces UPR and autophagic vesicles formation through PERK (Kouroku et al., 2007), whereas tunicamycin and tapsigargin require IRE1 (Ogata et al., 2006). The importance of UPR-dependent autophagy mediated by PERK or IRE1 in response to hypoxia is currently being investigated by many laboratories.

Regulation of apoptosis and survival. In addition to these four main effectors pathways that serves as a tolerance mechanisms, prolonged ER stress becomes toxic, and the UPR can promote apoptosis. The pro-apoptotic family members BAX and Bcl2 homologous antagonist/killer (BAK) associate with activated IRE1 and are required for signaling the transcriptional UPR response through XBP1 activation (Hetz et al., 2006). Interestingly, the transcriptional regulation of C/EBP

homologous protein CHOP, which is induced downstream of both PERK and IRE1, confers increased apoptotic sensitivity to at least some forms of ER stress (Zinszner et al., 1998). This may occur through the transcriptional regulation of CHOP-dependent targets such as ERO1L and GADD34 (Marciniak et al., 2004). As GADD34 expression restores protein synthesis by causing eIF2α dephosphorylation, it therefore increases the client load of proteins that must be properly folded within the ER. In the continued presence of agents that disrupt protein maturation this can increase their toxicity (Marciniak et al., 2004). Induction of ERO1L during ER stress could potentially catalyze disulphide bond in the ER, and by doing so, stabilize toxic unfolded protein aggregates.

1.3.3 Hypoxia and UPR signaling in tumors

Tumor hypoxia was first described in the 1950 by radiation oncologists as a frequent cause of failure to radiotherapy in solid tumor (Warburg, 1956). Today, it is evident that tumor hypoxia and the critical mediators HIFs, regulate multiple steps of tumorigenesis, including tumor formation, progression, and response to therapy.

Human cancers contain areas of necrosis in which cancer cells have died due to inadeguate oxygenation (Vaupel and Mayer, 2007). Cells closest to a perfused blood vessel are exposed to relatively high O_2 concentrations, which decline as distance from the vessel increases. Although such gradients exist in normal tissues, in cancer the gradients are much steeper (Iliopoulos et al., 1996), and O_2 concentrations drop to near zero in areas of necrosis. In addition to physical gradients, temporal fluctuations in oxygenation also commonly occur within tumors. Immunohistochemical analysis of cancer biopsies revealed increased levels of HIF1α and/or HIF2α proteins in the majority of primary human cancers and their metastases (Talks et al., 2000). Intratumoral hypoxia is a major mechanism underlying the increased levels of HIF1α and HIF2α in cancer and stromal cells. Other inducers of HIF1α in the tumor microenvironment include reactive oxygen and nitrogen species (Brunelle et al., 2005),(Kaelin, 2005), which also inhibit proteasomal degradation of HIF1α. Moreover, the activation of the phosphatidylinositol-3-kinase and MAP kinase pathways increases HIF1α synthesis (Mazure et al., 1997), primarily through the action of mTOR (Majumder et al., 2004).

HIF1α and HIF2α protein levels can also be increased in cancer cells due to loss of function of many different tumor suppressors, which results in either increased HIF1α synthesis or decreased HIF1α degradation (Maxwell et al., 1999) (Iliopoulos et al., 1996; Zelzer et al., 1998), (Zundel et al., 2000). Taken together these observations provide evidence that HIF1 activation promotes oncogenesis and/or cancer progression

To date the link between hypoxia and UPR has been studied mainly in the contest of cancer cells. Tumor cells have important interactions with the cellular context in which they are found including dependence on fundamental limiting factors such as lack of nutrient, oxygen and acidosis. These deficiencies can be partially overcome if the tumor is able to establish a blood supply via secretion of pro-angiogenic factors. In addition, hypoxia and nutrient deprivation inhibit N-linked

glycosylation of proteins, leading to inaccurate protein folding. Thus an important adaptive response for the tumor cell is the ability to overcome such limitations.
Overexpression of ER-resident chaperones Grp78 and Grp94 has been reported in several breast cancer cell lines (Gazit et al., 1999), overexpression of Grp78 has also been reported in colon cancer and gastric adenocarcinoma cell lines (Ramsay et al., 2005), (Song et al., 2001). Ex-vivo human tissues and animal models have reported an increase in Grp78 expression in malignant breast and colon cancer and a generalized activation of the UPR in hepatocellular carcinoma, gastric and oesophageal adenocarcinoma (Fernandez et al., 2000), (Shuda et al., 2003), (Chen et al., 2002)
It is known that activation of the PERK pathway can be important in mediating these effects. As discussed above, PERK activity leads to phosphorylation of the translation initiation factor eIF2α and suppression of protein synthesis from the vast majority of the mRNA present in the cells. Cells bearing genetic mutations that disrupt the function of PERK, or its downstream effectors eIF2α and ATF4, all show impaired survival and proliferation when challenged with low oxygen levels (Bi et al., 2005; Koumenis, 2006; Koumenis et al., 2002). These studies provide genetic evidence that PERK signaling can promote cell survival during hypoxia (Koumenis and Wouters, 2006).
Several studies have linked PERK signaling with enhanced tumor growth and survival under hypoxic conditions. Moreover, it has been reported impaired vasculogenesis and cancer cells proliferation in mice models were PERK signaling was genetically ablated. These studies suggest a model by which tumor cells manipulate PERK signaling to enhance viability by reducing translational activity, thereby reducing the metabolic demand, and promote tumor growth by increasing the production of angiogenic factors in response to low oxygen levels (Malhotra and Kaufman, 2007).
PERK is also involved in apoptosis induced by tumorigenesis through activation of CHOP. Increased CHOP levels have been reported within solid tumors, indicating that PERK signaling may be promoting apoptosis in these regions. However it is still unclear how cells balance the beneficial versus cytotoxic outputs derived from PERK signaling; in fact the attenuation of translation imposed by PERK, while providing protective benefits, would ultimately be detrimental if protein synthesis fell below levels necessary to sustain cellular functions (Koumenis, 2006). However area of central necrosis are often observed within rapidly growing solid tumors and it has been hypothesized they could represent gross manifestations of dynamic switching between the protective and toxic properties of PERK signaling.
XBP1 overexpression has been observed in colorectal carcinomas, adenomas and breast carcinoma. Functional studies of IRE1/XBP1 pathway could demonstrate its direct involvement in solid tumor growth. Cancer cells containing a homozygous deletion mutation of XBP1 gene showed impaired survival under hypoxia (Romero-Ramirez et al., 2004). The $XBP1^{-/-}$ cells also failed to form tumors in animals. Experiments using a human tumor xenograft expressing an XBP1s-luciferase fusion protein, demonstrated the expression of spliced active XBP1s in all tumors including small ones, suggesting that the ER stress persists throughout tumor growth (Spiotto et al., 2010). Activation of the third ER stressor ATF6 by hypoxia has not been demonstrated but is probable, based on the information that many of its transcriptional targets are induced under hypoxic conditions. Taken

together these results demonstrate an important role for the UPR in determining hypoxia tolerance and tumor growth.

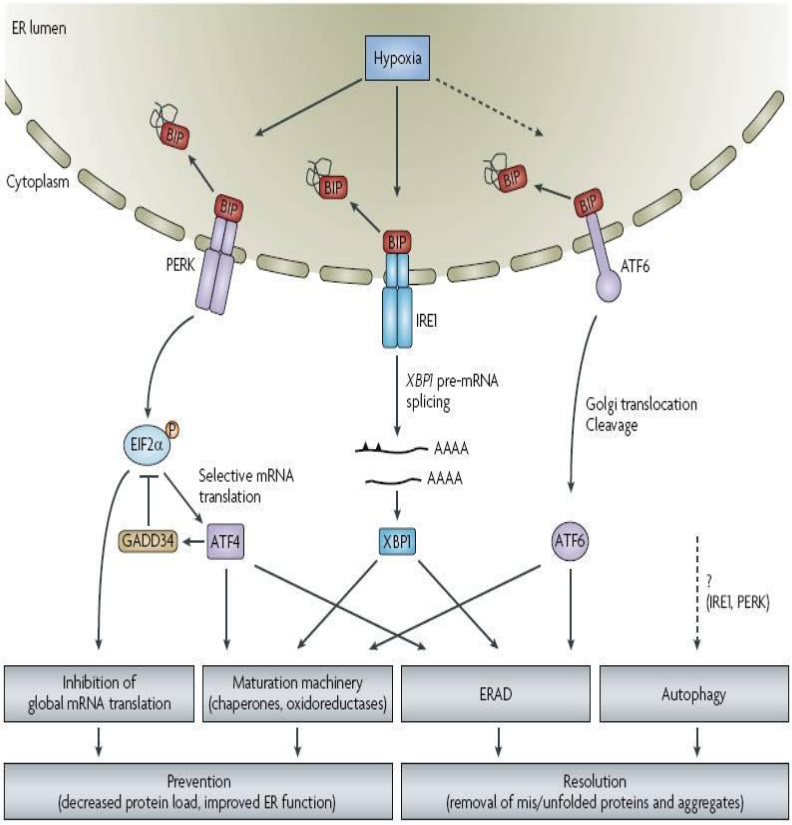

Figure 9. Hypoxia activates the UPR*. The ER stress sensors are activated during ER stress through loss of binding to BiP and/ or direct interaction with misfolded proteins and aggregates. PERK activation results in inhibition of global mRNA translation. A transcriptional response also occurs through activation of the transcripton factors ATF4, XBP1 and ATF6. Hypoxic activation of ATF6 has not been directly demonstrated, but is probably based on hypoxic induction of ATF6-dependent genes such as BiP and GRP94 and Derlin3 (our observations). ATF4, XBP1 and ATF6, induce an overlapping set of proteins that increase the capacity for ER-localized proteins maturation and ER-associated degradation (ERAD). The molecular pathways that lead to ER-stress induced autophagy remain unknown, but are dependent on PERK and/or IRE1. The basis for how hypoxia causes ER-stress and activation of the UPR is not yet fully understood. From Wouters et al. Nature Reviews Cancer 2008.*

Since enhanced survival of tumor cells under hypoxic conditions mediated by PERK and IRE1/XBP1 have been observed in established tumors, that raises the intriguing questions regarding whether such cells have a selective prosurvival advantage that contribute to the process of advanced carcinogenesis. No data are available about the role of the UPR on cancer initiation. Moreover many experimental data, with simulated hypoxic conditions, have been documented in cultured cells grown in anoxia.

2.SCOPE OF THE THESIS

2.1 Rationale

The protein components of eukaryotic cells face acute and chronic challenges to normal folding, refolding and function owing to a constant barrage of physical, metabolic and environmental stresses. The endoplasmic reticulum (ER) responds to the accumulation of unfolded proteins in its lumen (ER stress) by activating intracellular signal transduction pathways, cumulatively called the unfolded protein response (UPR). The UPR activation triggers an extensive transcriptional and translational response, which adjusts the ER protein folding capacity according to needs. As such, the UPR constitutes one of the signaling pathways that regulates the capacity and composition of the ER according to the folding capacity.
The experimental work described in this thesis aims at delineating the ER homeostasis by analyzing the heterogeneity of stresses and the fine tuning of the UPR signaling in different cellular conditions. My initial works studied the changes of the ER homeostasis when cells overexpress secretory proteins and during plasma cell differentiation (Chapter 1.2). Recent studies have indicated that the cells suffering from insufficient blood supply experience stress. The ER needs energy and oxygen (O_2) for the folding process, thus nutrient deprivation and hypoxia, caused by insufficient oxygen, lead to inefficient protein folding and ER stress. Hypoxia alters the behavior of the cells through different O_2 sensitive pathways, the best understood is the one mediated by the hypoxia inducible factor family of transcription factors (HIFs). Recent *in vitro* studies have shown that the hypoxic stress triggers the UPR. However, *in vivo* and *in vitro* situations are indeed likely to diverge substantially with respect to parameters such as metabolic activity, O_2 utilization and cell division rates, features that are predicted to vary the cell sensitivity to ER stress. Thus, despite a recognized role for hypoxia on UPR, few data exist on the effects of hypoxia in various organs *in vivo*.

2.2 Aims

Our study includes three aims:

- First we will test if the hypoxic stress *in vivo* acts as a modifier that affects the activation of specific branches of the UPR in different tissues.
- Second, to get a better insight into the role of the UPR during low oxygen availability in tissues, we test whether the UPR activation depends from the severity of the hypoxic stress.
- Third we aim at delineating signaling circuits that control the capacity and composition of the proteostasis network through transcriptional and post transcriptional mechanisms to balance the ER homeostasis

2.3 Strategy

To test the UPR dependence on stress severity, we expose mice to two levels of O_2 reduction for 5 h, a time frame that allows induction of mRNA transcripts before the setting of adaptive mechanisms. We give priority to liver and myocardium tissues because they are homogeneous tissues and widely differ in terms of O_2 availability and requirement. Comparison of several UPR markers enable to underscore the ability to selectively activate different arms of the UPR.

3.MATERIALS AND METHODS

3.1 Animals.

We used male ICR CD-1 mice (5-weeks old, 29.6±0.3 g body weight at entry into the study). Animals were placed in the hypoxic chambers (Corno et al., 2002) and exposed to either 6.5% O_2 (severe hypoxia), 10% O_2 (moderate hypoxia) or 21% O_2 (control) for 5 h (n=10/group). All animals had free access to water and conventional laboratory diet until 24 h before sacrifice. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). At the end of hypoxia exposure, animals were transferred one-by-one into the procedure chamber kept at the same $\%O_2$ as the hypoxic chamber, anesthetized by i.p. Na-thiopental (10 mg/100 g body weight) plus heparin (500 units) and sacrificed by cervical dislocation. Only after sacrifice, the animals were taken out of the chamber and dissected, heart and liver were rapidly excised and immersed in liquid nitrogen, the whole procedure taking <1 min after sacrifice. Thus, all organs were taken during hypoxia preventing reoxygenation. Organs were stored at -80°C until use.

3.2 Isolation of RNA and quantitative real–time PCR.

Total RNA was isolated using TRIZOL reagent (InVitrogen) according to the manufacturer's recommendations. Reverse transcription was performed using 2 μg of total RNA, oligo d(T) primer and 50 Units of AMV Reverse Transcriptase (InVitrogen). Quantitative PCR was performed using real-time detection technology and analyzed on a ABI prism 7500 Sequence Detector (Applied Biosystems) and relative quantification of gene expression was performed using the comparative threshold (C_t) method as described by the manufacturer. Power SYBR green PCR master mix (Applied Biosystems) and gene specific primers were used to analyzed mRNAs expression. The mouse actin beta gene was used as internal control to normalize the mRNA levels for each gene. Primers sequences are reported in Table1.

	Table 1	
name	**sequence 5'-3'**	**PCR bp**
mCHOP for	GTGCATCTTCATACACCACCA	
mCHOP rev	CTCCTGCAGATCCTCATACC	185
mGADD34for	CTTCGCGAGCAGTCCGGA	

mGADD34rev	GACAGGAGATAGAAGTTGTGG	159
mDerlin3 for	GCCACATCTACTACTTCCTAG	
mDerlin3 rev	CAGAGTGGGCTTCCTGCTC	184
mXBP1 for	CCTTGTGGTTGAGAACCAGG3	
mXBP1 rev	CTAGAGGTTGGTGTATAC	530
mActinb for	CACCCACATAGGAGTCCTTC	
mActinb rev	CAGTTCGCCATGGATGACG	171
mTRB3 for	CGAGTGAGAGATGAGCCTGA	180
mTRB3 rev	CCCCATGGGTCTTCGTGAAA	

XBP1 splicing reaction was analized as published (Yoshida et al. 2003). XBP1 PCR products were separated on 2% Nusieve3:1 agarose gel, followed by ethidium bromide staining and densitometric quantification of band intensities. Bands intensities were measured by the Gel Doc system.

3.3 Western Blotting

Frozen tissues samples were lysed in potter homogenizer with a buffer containing 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT 0.2 mM PMSF, protease inhibitor cocktail (Sigma), 1 mM NaF, 100 nM Na orthovanadate. Samples were spun at 1200 rpm for 10 min to pellet nuclei. Nuclei were lyzed with a buffer with 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5mM DTT, 0.2 mM PMSF, protease inhibitor cocktail, 1mM NaF, 100 nM Na orthovanadate. Protein concentration was measured at 492 nm using the Coomassie Blue Reagent (Sigma).
For the blot, 50 µg of lysate was separated on 10% PAGE under reducing conditions. Proteins were transferred to nitrocellulose filter (Amersham Biosciences) and blotted with p54-JNK, total-JNK, Bcl2 , Bax and Tubulin (Santa Cruz Biotecnology) antibodies. Proteins were visualized with ECL detection system (Amersham).

3.3 TUNEL assay

Apoptosis was assessed using the *In Situ* Cell Death Detection kit, TMR red (Roche), where the 3'-OH DNA ends, generated during DNA fragmentation, were labeled with TMR red-nucleotides by the terminal deoxynucleotidyl transferase (TdT). The nuclei were then counterstained with Hoechst 33258 (Sigma).The slides were examined on a magnification of x 40 in an inverted fluorescence microscope (Axiovert 25 CFL, Zeiss, Gottingen, Germany) equipped with a filter for

the detection of rhodamine (filter set 15, excitation bandpass 546 +/- 12nm, emission 590 nm), or with a filter for Hoechst staining (Filter set 02, excitation bandpass 365, emission 420). The images were acquired by a digital camera system for microscopy (DS-5M); Nikon, Tokio, Japan). Two operators counted the number of TdT-labeled and total nuclei by examining at least 5 random fields in a blinded procedure. Results are expressed as number of TdT labeled nuclei/total nuclei/0.037 mm^2.

3.3 Statistics

All data are expressed as mean±SEM. To assess significant differences among the groups, we first performed one-way ANOVA, then the Bonferroni multiple comparisons test if ANOVA was significant. The significance level was set to $p<0.05$.

4. RESULTS

4.1 Acute hypoxia activates UPR in liver and myocardium

To test whether the hypoxic insults activate the UPR pathway in vivo, mice were kept for 5 h either under 6.5% O_2 or 10 % O_2. All animals survived the treatment. Total RNA from liver and myocardium was extracted and the level of mRNA transcripts of CHOP and GADD34, which are known to be induced during ER stress in vitro and in vivo were measured. In liver tissue, the levels of CHOP and GADD34 mRNA were induced 1.5 and 2,3 times respectively under 6.5% O_2. In myocardium tissue, the levels of CHOP and GADD34 mRNAs did not change by hypoxia in vivo (Figure 10A and B). Next we measured the mRNA expression level of the human homolog of Drosophila tribbles (TRB3). TRB3 is induced by various form of endoplasmic reticulum stress as a part of the UPR signaling response (Jousse et al., 2007). In hepatocytes TRB3 was expressed under normal conditions and its expression was augmented by 10% and 6.5% O_2 by 4.7 and 12.6 folds respectively. TRB3 was not expressed in steady state myocytes but was highly induced by 10% O_2 (10 folds) and 6.5% O_2 (92 folds), (Figure 10C).

The level of Derlin-3, a component of the UPR pathway that is upregulated by accumulation of unfolded proteins and that promotes ER-associated degradation (ERAD), was induced at similar extent in liver and myocytes of mice subjected to 6.5% O_2 (4.3 and 4.8 folds respectively, Figure 10D). As a whole, these results indicate that myocardium and liver tissues respond to limited O_2 availability by activating the UPR genes. Therefore the UPR response to *in vivo* hypoxia is tissue-specific and depends on the stress severity.

We could not detect changes in eIF2α phosphorylation in hepatocytes and myocytes after hypoxic exposure at 10% and 6.5% O_2 (data not shown). It has been reported that small changes as 20% in the phosphorylation levels of eIF2α can dramatically suppress translation initiation (Kaufman, 2002), (Koumenis et al., 2002). Moreover, it has been shown, using different cell culture systems, that severe hypoxia (<0.5% O_2) causes a rapid phosphorylation of eIF2α, followed by a partial recovery after 4-6 hours, consistent with the activation of a negative feedback loop. (Koumenis et al., 2002), (Koritzinsky et al., 2006). In contrast phosphorylation of eIF2α during moderate hypoxia (1% O_2) requires long hypoxic exposures. In order to check for the activation of the PERK-dependent axis of the UPR we detected the expression level of the transcription factor ATF4 which is translationally upregulated in an eIF2α phosphorylation dependent manner. Both 10% O_2 and 6.5 % O_2 increase the expression of ATF4 in hepatocytes (Figure10E) consistent with the expression of CHOP.

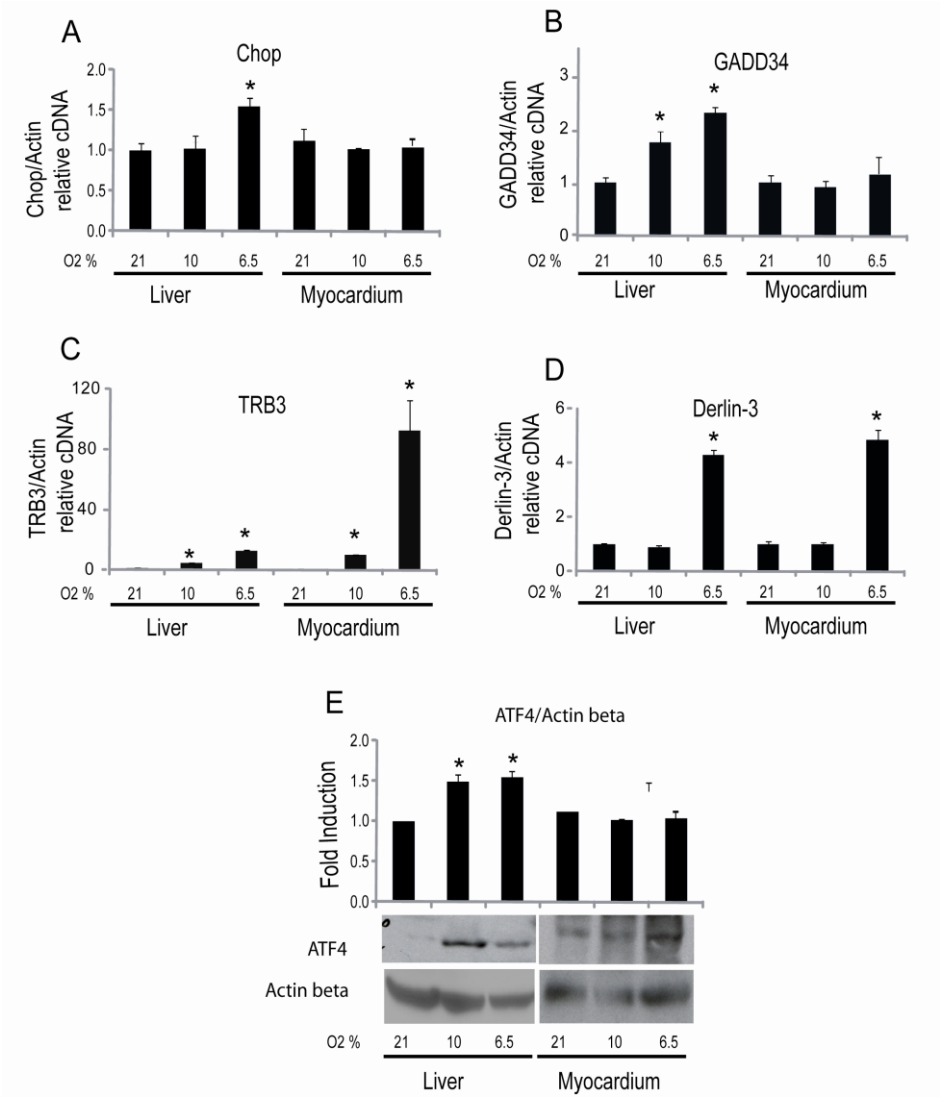

Figure 10. Upregulation of the UPR markers in liver and myocardium tissues of mice exposed to hypoxia at the indicated % O_2 for 5 h. A-D. *Quantitative Real Time RT-PCR for CHOP, GADD34, TRB3 and Derlin3, β-actin gene cDNA is used as internal control. The graphs represent the average and standard deviations of triplicate real time reactions of two animals for each treatment. One-way ANOVA* p<0.05 vs. 21%O_2.* ***E.*** *Tissues lysates were separated in 5-15% PAGE and blotted with anti ATF4 and β actin specific antibodies. Graphs represent densitometry analysis of bands compared to protein levels in normoxic animals.*

4.2 Acute hypoxic stress results in increased apoptosis, JNK phosphorylation and decreased Bcl2

The TUNEL assay showed clear signs of DNA fragmentation in liver and myocardium after acute hypoxia (Figure 11). Both 10% and 6.5 % O_2 resulted in 10-20 fold increase in the number of TUNEL-positive nuclei in liver and myocardium tissues.

Because activated IRE1 has been shown to interact with the adaptor protein TRAF resulting in activation of apoptosis through C-Jun amino terminal kinase (JNK), we measured the level of JNK phosphorylation in the cytosolic fractions of hepatocytes and myocytes after the 5 h hypoxic stress. The quantitative densitometry of the blots shows that in liver tissue the level of p54-JNK protein was upregulated 1.5 fold in mice exposed to 6.5% O_2 (Figure 12). The level of the anti-apoptotic protein Bcl2 was reduced by 40% and the Bcl2/Bax ratio was also reduced. In myocardium tissue, the level of p54-JNK protein was unaffected by hypoxia. At 6.5% O_2 Bcl2 decreased by 50% and the ratio Bcl2/Bax was reduced at 50% as well. This shows that hypoxic stress changes the balance between pro-apoptotic and anti-apoptotic proteins in tissue-specific manners.

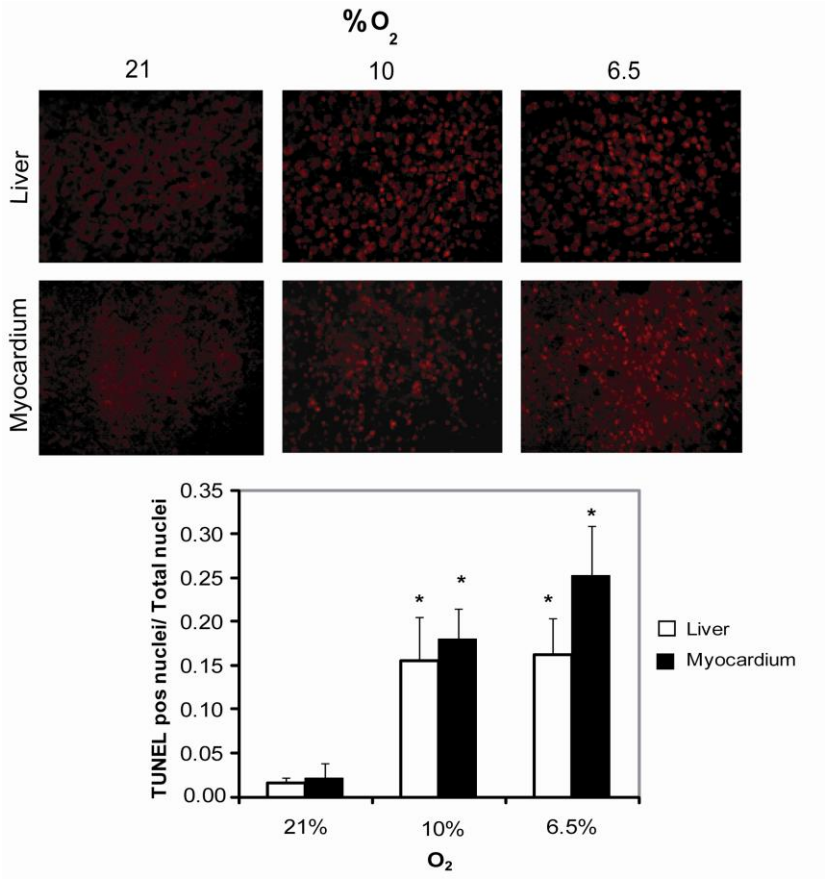

Figure 11. Apoptosis by TUNEL assay of myocardium and liver tissues of mice exposed to *hypoxia at the indicated % O2 for 5 hours. The apoptotic nuclei are dyed with tetramethyl- rhodamine. The graph indicates the ratio between TUNEL positive nuclei and total nuclei number in 0.037 mm2 (mean±SEM, n=6 per group). One-way ANOVA p<0.001 and 0.002 for liver and myocardium, respectively. * p<0.05 vs. 21%O2 (Bonferroni post-test).*

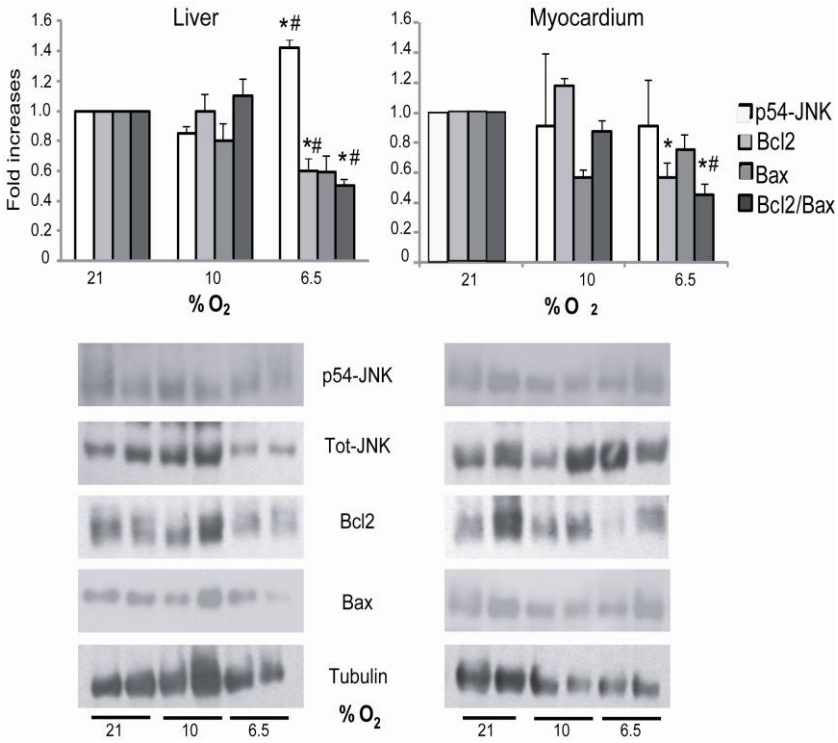

Figure 12. Effect of 5 h hypoxia on apoptotic proteins in liver and myocardium*. Tissues lysates were separated in 10% PAGE and blotted with specific antibodies. Graphs represent densitometry analysis of bands compared to protein levels in normoxic animals (mean±SEM, n=6 per group). One-way ANOVA p<0.01 and 0.02 for liver and myocardium, respectively. * p<0.05 vs. 21%O_2, # , p<0.05%vs.10% O_2 (Bonferroni post-test),*

4.3 Hypoxia does not activate XBP-1-splicing

The IRE-1-dependent branch of the UPR involves splicing of 26 nucleotides from the mRNA of the XBP-1 gene (Bertolotti and Ron, 2001; Lee et al., 2002; Liu et al., 2002). In turn, this event results in translation of the XBP-1 gene to produce a factor that regulates the transcription of proteins involved in the UPR signaling pathway (Urano et al., 2000). We investigated the effect of hypoxia on XBP-1 mRNA splicing using a PCR-based assay. Figure 13 shows that the level of spliced XBP-1 was undetectable in both liver and myocardium tissues of mice treated with either 10% or 6.5% O_2 for 5 h indicating that hypoxic stress does not induce XBP-1 splicing.

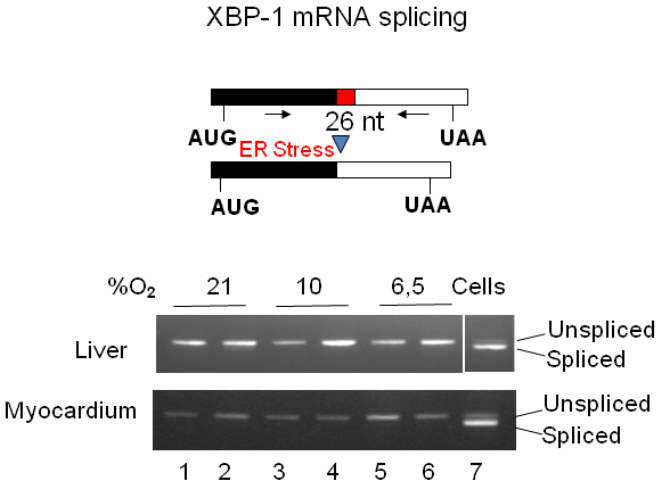

Figure 13. Analysis of XBP-1 splicing in liver and myocardium from mice exposed to 10% or 6.5% O_2. The PCR products of two samples per group are shown. As a control, HEPG2 cells were incubated for 5 h with 5 µg/ml of tunicamicin, a strong inducer of the UPR.

5. DISCUSSION

5.1 Unfolded proteins and hypoxia

We studied the role of hypoxia on UPR signaling pathway *in vivo.* Giving the heterogeneity of oxygen availability in organs, we hypothesized that different tissues may respond to hypoxic insults by activating specific arms of UPR signaling. At the same time we aimed to use more physiologically relevant models than the early literature and paid special attention to study the molecular events and signaling pathways activated when animal are exposed to low O_2 levels.
Our overall results are consistent with the literature reports on activation of the UPR in cell culture grown under hypoxic conditions, at the same time give new insights about the oxygen-dependent activation of specific branches of the UPR in different tissues.
In this study, animals were exposed to varying degrees of hypoxia and sacrificed without reoxygenation. Therefore, the presented data are free of possible artifacts due to the presence of reactive O_2 species that may disturb the UPR pathway, and the direct effect of hypoxia is thus emphasized. As hepatocytes and cardiomyocytes are from relatively homogeneous organs, whole cell and RNA extracts well represent the behavior of the organ to which they belong. Whereas the myocardium is an organ with high O_2 demand that quickly overexpresses HIF-1α within 1 h of hypoxia (Caretti et al., 2007), the liver is perfused for 80% with low-PO_2 portal venous supply. Thus, by ranging from 44.4±5.1 mmHg in sinusoids to 4.5±1.3 mmHg across the lobus (James et al., 2002), liver tissue PO_2 is rather low, and the liver may be considered a permanently hypoxic organ that has dismissed its ability to accumulate HIF-1α (Bianciardi et al., 2006). Therefore, liver and myocardium tissues widely diverge for their O_2 metabolism and can be taken as extreme paradigms that encompass most of the other organs.
Our experiments show that 6.5% O_2 reduction results in induction of CHOP and GADD34 transcripts, and translation of ATF4 downstream of the PERK-axis (Figure 10) in hepatocytes. Severe hypoxia in liver results also in activation of the cell death signaling pathway dependent from the IRE1 branch of the UPR, as the increase of p-JNK (Figure 13). It has been known that upregulation of CHOP promotes cell death (McCullough et al., 2001), whereas CHOP deletion protects against the death of ER stressed cells (Zinszner et al., 1998), (Oyadomari et al., 2002). These observations suggest that CHOP evolved to link insurmountable levels of ER stress to the cell-death machinery. Furthermore, CHOP has been reported to inhibit the expression of Bcl2 (McCullough et al., 2001) and directly activate the proapoptotic death receptor 5 protein, which recruits procaspase-8 to form a death-inducing signaling complex. IRE1 has been reported to induce the proapoptotic signaling via its kinase domain, which can activate the adaptor protein TRAF2 and the downstream effector kinase p-JNK (Nishitoh et al., 1998) (Nishitoh and Ichijo, 2004).

All together these results suggest that the liver is able to activate the PERK-dependent arm of the UPR during hypoxia in order to balance both the attenuation of protein synthesis and apoptosis. Liver and myocardium tissues have different rate of protein synthesis, being higher in hepatocytes, that are able to produce elevated levels of secretory proteins, than in myocardium. However, while the liver can regulate protein synthesis and inhibition according to its own physiological needs and energy demands, the myocardium is resistant to suppression of protein synthesis (Yuan et al., 2008).

TRB3 is a UPR-induced gene whose expression is upregulated by CHOP and ATF4 transcription factors. Moreover, experiments of overexpression of TRB3 have shown that is able to reduce the levels of CHOP and GADD34 mRNA (Ord et al., 2007), suggesting that TRB3 acts as a negative feedback regulator of the ATF4-CHOP dependent transcription (Ohoka et al., 2005), (Jousse et al., 2007), (Su and Kilberg, 2008). Although the exact mechanism by which TRB3 inhibits the transcriptional activation of ATF4 remains obscure, it has been proposed that TRB3 inhibits ATF4 and CHOP by interfering with the recruitment of cofactors of the transcriptional apparatus.(Ord et al., 2007)

Since CHOP and ATF4 are induced by many ER stress signals, as oxidative stresses, amino acid deprivation and hypoxia, it is possible that the CHOP-TRB3 pathway operates in response to these stresses as well. Indeed, it has been reported that TRB3 is induced in *in vitro* models of hypoxia and ischemia (Bowers et al., 2003), (Avery et al., 2010). Here we show that TRB3 expression is significantly upregulated by 10% and 6.5 % O_2 *in vivo* in liver and myocardium and is a sensitive marker of hypoxic-stress, however its function in hypoxic tissues remains to be clarified. It has been proposed that TRB3 could be the sensor for ER stress-induced apoptosis. If the ER stress is transient and mild, the induced TRB3 blocks the CHOP and ATF4 functions by binding to them, however, when potent and prolonged ER stress occurs, TRB3 leads to apoptosis (Ohoka et al., 2005).

Derlin 3 is an ATF6-regulated protein member of the Derlins family genes. Like the homologous Derlin 1 and 2, Derlin 3 is induced by ER stress in several cell types (Oda et al., 2006). In a recent study it has been found that Derlin 3 was induced by ATF6 *in vivo* and in cultured cardiac myocytes. Derlin3 was also induced in the infarct border zone in an *in vivo* mouse model of myocardial infarction and published results suggest that Derlin 3 may have a protective role in the heart (Belmont et al., 2010). In our experimental conditions Derlin 3 is induced at similar extent in liver and myocardium by hypoxia. Moreover, to our knowledge this is the first report of upregulation of Derlin 3 under hypoxic stress. Since Derlin 3 transcription has been shown to be under the control of ATF6 (Belmont et al., 2010), our results suggest that the ATF6 branch of the UPR is activated by hypoxia, although further in vitro experiments must confirm our finding.

Our *in vivo* experiments could not detect XBP-1 splicing in liver and myocardium after 5 h of O_2 reduction, independently from the severity of the hypoxic insult. Attempts to understand the time frame of UPR pathway activation has elucidated that mammalian cells are able to execute a time-dependent phase-transition from protein translational attenuation to protein degradation depending on the quantity of misfolded proteins accumulated in the ER (Yoshida et al., 2003). It is possible that the level of accumulated misfolded proteins after hypoxic insults does not reach a threshold necessary for activating this arm of the UPR. To corroborate our

finding, XBP1 splicing has been detected in tumor cells grown *in vitro* only under severe hypoxia (<0.1% O_2) (Koumenis et al., 2002).
Given the detection of CHOP and JNK activation in liver it seems that the activation of the PERK-dependent signaling pathway is the first answer to acute hypoxia in liver. These results suggest that upon UPR stress hepatocytes (which are self renovating cells) balance between attenuation of translation and apoptosis. The TUNEL assay highlights the multitude and complexity of apoptotic pathways activated when oxygen availability becomes low, some of them can be traced to ER stress while others may be activated by different insults (Xu et al., 2005).
From the data illustrated in this study and from literature reports, we can propose the following scheme shown in Figure 14 where 6.5% O_2 reduction activates all the three branches the UPR pathway in liver. While the hepatocytes activate the apoptotic pathway mediated, in part, by CHOP and p-JNK, we could not detect an UPR-dependent apoptosis in myocytes, moreover in the myocardium the ATF4-CHOP-GADD34 signaling pathway is not detectably activated and the protein synthesis remain sustained under low oxygen availability.

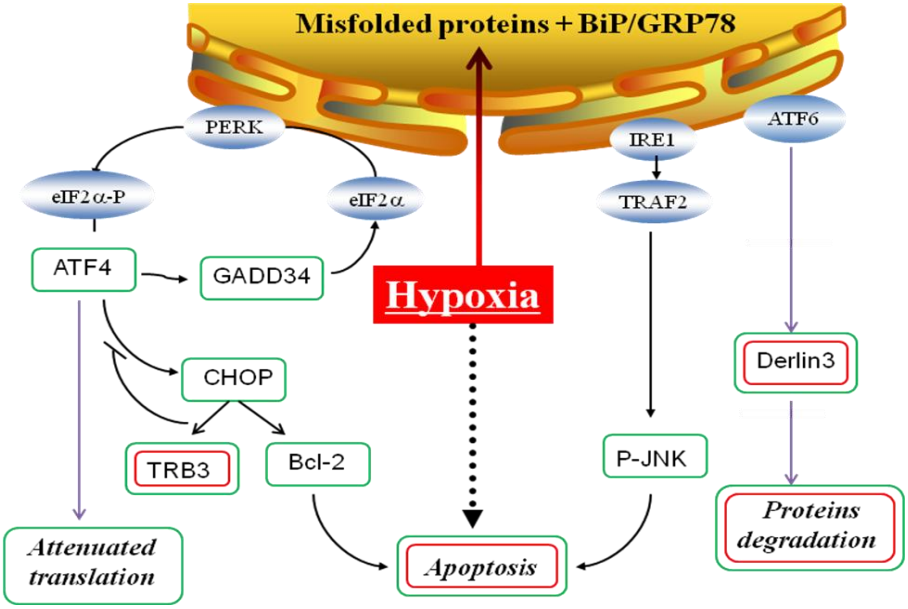

Figure 14. Activation of the unfolded proteins response (UPR) pathway following hypoxic stress. Upon accumulation of misfolded proteins, the BiP/GRP78 is released from cognate transmembrane receptors. As a first consequence PERK is activated and phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) on Ser[51]. The transcription factor CHOP, the anti apoptotic protein Bcl2, the phosphatase GADD34 and TRB3 are activated. The inositol-requiring protein 1 (IRE1) cytoplasmic domain interacts with the adaptor protein TRAF2 that couples c-Jun activation linking ER stress to apoptosis. The transmembrane UPR-sensor ATF6 is cleaved and the cytoplatsmatic portion, liberated from its transmembrane tether, can bind to DNA and promote transcription of ER associated degradation genes as Derlin 3. This activation cascade is modulated by the intensity of ER stress and, as

consequences, by the amount of misfolded protein accumulated. Genes and effectors pathways activated in liver are depicted in green, in red when activated in myocardium.

5.2 Toward a model of proteostasis network

Understanding how the unfolded polypeptides, resulting from the process of translation, arrive at their native structures for function and how these structures are maintained and ultimately turned over is a major challenge in biology. Furthermore we need to progress toward translating these insights into meaningful improvements in the diagnosis and treatments of many diseases associated with protein misfolding. Unlike protein folding in a tube, a protein in a eukaryotic cell must fold and function in a crowded environment. Moreover proteins are subjected to extensive changes in structure, as they cycle between inactive and active conformations, and/or they engage in the protein-protein interactions. We now recognize that the chemical and energetic properties specified by the amino acid sequence of a polypeptide, (the primary structure), encoded by the genome, while very important in partially determining the folding energy landscape, are only part in determining how proteins evolve biological functions. Numerous macromolecular assistants exist in the cells to influence the folding of the proteome, such assistance controls the rate of protein synthesis, influences the rate of folding, affects membrane trafficking and compartmental localization, mitigates aggregation and mediates degradation, enabling protein turnover. These competing biological pathways, comprising hundreds of components, make up the proteostasis (protein homeostasis) network (Balch et al., 2008).

Proteostasis refers to controlling the concentration, conformation, binding interaction (quaternary structure), and location of individual proteins making up the proteome by readapting the innate biology of the cell, often trough transcriptional and translational changes. The proteostasis is influenced by the chemistry of protein folding /misfolding and by numerous regulated networks of interacting and competing biological pathways.

The proteostasis network and its pathways are controlled by numerous integrated signaling events that optimize the capacity of the proteostasis to network and maintain protein function. This occurs by more efficiently folding the natively folded proteins and maintaining the unstructured cellular proteins in a non aggregated state. The proteostasis network is ancient, furthermore coevolved with the remarkable diversity of polypeptide sequences to enable the evolution of a wide variety of organisms by expanding the capacity of proteins to function in increasingly complex cellular and subcellular environments, and to perform increasingly specialized cellular tasks.

It is the job of the cellular proteostasis network to enable cellular protein folding and function, in the face of all the challenges discussed above. The proteostasis network consists of numerous biological pathways that comprise chaperones, folding enzymes, degradation and trafficking components. Control of the proteostasis network is accomplished by signaling pathways that directly regulate the concentration, distribution and activities of the different components of the

proteostasis network itself (Morimoto, 2008), (Young et al., 2004). Different cells have varying proteostasis capacities reflected in the composition and concentrations of their proteostasis components, presumably to handle the different folding challenges that arise in response to differentiation during development.

Numerous factors influence the properties of the proteostasis network including changes in intracellular ATP levels, amino acids pools, metabolites, lipid homeostasis, and ion balance. These not only alter folding capacity, but also modulate the activity of a number of other proteostasis network pathways such as the degradative pathways, protesomal, lysosomal and autophagic, that are integral to maintenance of proteostasis (Konstantinova et al., 2008), (Tasaki and Kwon, 2007), (Levine and Kroemer, 2008), (Kundu and Thompson, 2008).

The experimental work described in this thesis aimed at delineating the proteostasis network of the ER by analyzing the heterogeneity ER of stresses and fine tuning of the UPR in different cellular conditions.

In **chapter 1.2.1** we elucidated the relationship between unfolded protein accumulation in the ER lumen and ER stress, by analyzing the folding requirements and chaperones interaction of coagulation FVIII a large glycoprotein that is known to be prone to misfolding in the ER lumen even in its wild type form. Its expression provides a unique approach to study and manipulate the ER stress response that does not rely on pharmacological intervention.

Our results support that the level of cellular energy is important for protein folding and for disaggregation of transient aggregates within the ER. By contrast, in our hands, inhibition of proteasomal degradation did not result in accumulation of high molecular weight FVIII aggregates within the cells, suggesting that the active proteasome is not required for their removal and that these transiently formed aggregates may be rescued for secretion.

The proteostasis network is not only highly adaptable, enabled by the influence of multiple cell stress signaling pathways, but also can be quite distinct in each cell type. This is not surprising giving the central role of proteins in cell physiology and the diversity of cell functions. The response to diversification includes proteostasis network components that are conserved throughout evolution and those that are specialized. For example, hepatocytes, plasma cells and β cells must produce high levels of distinct secreted proteins, whereas fibroblasts have less secretory activity, and therefore a less specialized ER proteostasis network capacity. That suggests that a protein ability to achieve its functional state is dependent on the proteostasis network. Thus any effort to understand protein folding, and therefore protein function in vivo, will need to consider the interdependence between the folding energetic and the proteostasis network.

In **chapter 1.2.2** we studied the plasma cells differentiation and death, which involves the stepwise synthesis of different classes of proteins as to increase the secretory capacity and thus the proteostasis network of the cell. In particular, our studies have investigated the contribution of UPR signaling and degradative pathways to B-cell development. Our and other's results underline the role of the UPR signaling and proteasomal degradation in orchestrating the architectural and functional changes during terminal plasma cell differentiation.

The use of proteasome inhibitors in clinical approach illustrates the value of manipulating the proteostasis boundary to promote cell death. Proteasome

inhibitors decrease the degradation rate of misfolded proteins thus resulting in increased concentration of misfolded proteins, that likely saturates the proteostasis network, so that cells cannot sustain cell viability. In multiple myeloma, the proteasome inhibitor Boterzomib selectively kills the cancerous plasma cells that produce large amounts of immunoglobulin. This selectivity arises because myeloma cells are already challenged with a high protein folding load, and proteasome inhibitor further diminishes the capacity of the proteostasis network to maintain cells functional. Our results suggest that the unfavorable load-capacity ratio in Ig-secreting cells could make the latter more prone to enter apoptosis, either basally or following treatment with proteasome inhibitors. Moreover, our findings have important implication related to the potential utilization of proteasome inhibitors in the treatment of inflammatory disorders, especially those related to excessive antibody production.

Activation of ER stress sensors after hypoxic insults strongly suggests that lack of O_2 perturbs ER homeostasis. Although the direct requirement for O_2 in ER function remains to be clarified in detail, the ER has emerged as a cellular compartment that depends on O_2 for oxidative folding of secretory and transmembrane proteins and that mediates the O_2 signaling that is important for the survival and function of hypoxic cells.

In **chapter 4** we analyzed the effect of hypoxia as a physiologic stressor that affects the proteostasis in vivo. We considered the possibility that changes in the cellular oxygen availability correspond in changes in the proteostasis network components.

Both hepatocytes and myocytes respond to hypoxia by increasing their degradation activity as to increase the proteostasis capacity of hypoxic cells. At the same time hepatocytes are able to activate the PERK-dependent axis of the UPR that results in attenuation of protein translation.

Attenuation of protein synthesis following hypoxic insults is primarily due to a HIF-independent oxygen sensing pathway that directly inhibits the initiation step of mRNA translation. Inhibition of translation during hypoxia is important for its ability to both promote cell survival and to alter gene expression and cell phenotype. A general suppression of mRNA translation can also induce important changes in protein expression, due to differences in protein half-life, and allow to reset the proteostasis network. Furthermore, proteome changes are expected due to the fact that not all the mRNA transcripts are affected to the same degree. Finally, because translation efficiency can be rapidly modulated, this mechanism allows for much faster protein turnover that is possible through changes in transcription. Experiments performed with cell lines exposed to severe ($<0.05\%$ O_2) and moderate (0.2-1% O_2) hypoxia have shown inhibition of protein translation through eIF2α phosphorylation and thus dependent from the activation of the PERK kinase which regulates a branch of the UPR signaling pathway (Koumenis et al., 2002).

Interestingly, the ability to attenuate protein synthesis through the eIF2α-dependent phosphoryltion and translation of ATF4 appears to be a prerequisite of hepatocytes One of the fundamental differences between liver and myocardium tissues is the rate of protein synthesis, being higher in liver, that is able to produce elevated levels of secretory proteins, than in myocardium. However, while the liver can regulate protein synthesis and inhibition according to its own physiological

needs and energy demands, the myocardium is resistant to suppression of protein synthesis.
Although activated independently growing evidence suggest that HIF, and UPR-dependent response to hypoxia act in an integrated way, influencing each other and generating different signaling outputs that can be tailored to various cell types and physiological situations in order to modify the cellular proteostasis network. Further experiments must be performed using different transgenic models in order to understand which proteins are selectively induced during hypoxic insults when mechanisms of translational attenuation are activated.

6. CONCLUSIONS

In conclusion our studies could underline several features of the ER- homeostasis

- First the level of oxygen availability can influence the proteostasis capacity of the ER *in vivo* by activating some branches of the UPR signaling pathway.
- Second the proteostasis network is adaptable and able to fine tune the UPR signaling pathway in response to the stress intensity. Moreover different cells have varying proteostasis capacities reflected in the composition and concentrations of their proteostasis components
- Third, the cellular energy levels affect protein folding as well as protein disaggregation and thus mediate folding and repair process. The proteasome activity, by mediating protein degradation, can balance the proteostasis capacity of the cell.

This work also illustrates the complexity of the proteostasis network for cell homeostasis. The cellular capabilities to manage excess of protein folding load are limited and therefore, by setting the proteostasis boundary as a threshold for generating folded and functional proteins, the proteostasis network can create and maintain functional proteins in response to the local environment.

6.1 Implications

Signaling pathways that regulate protein synthesis, folding, trafficking, aggregation, disaggregation and degradation include the UPR, which principally influence the ER folding capacity (Malhotra and Kaufman, 2007), (Ron and Walter, 2007), the heat shock response (HSR), which balance proteostasis capacity in the cytosol (Shamovsky and Nudler, 2008), pathways that influence subcellular Ca^{++} concentrations (Petersen et al., 2005), (Burdakov et al., 2005), the inflammatory response, which regulate cell defense (Medzhitov, 2008), (Zhang and Kaufman, 2008) and histone deacetylase (HDAC), which integrate proteostasis capacity through epigenetic pathways (Fig 15). Such signaling events control the capacity and composition of the proteostasis network through transcriptional, translational and posttranslational mechanisms to balance proteostasis by reducing demand, enhancing folding and repair processes, and mediating degradation. When proteostasis is severely compromised, cell death pathways are activated targeting the cell for destruction.

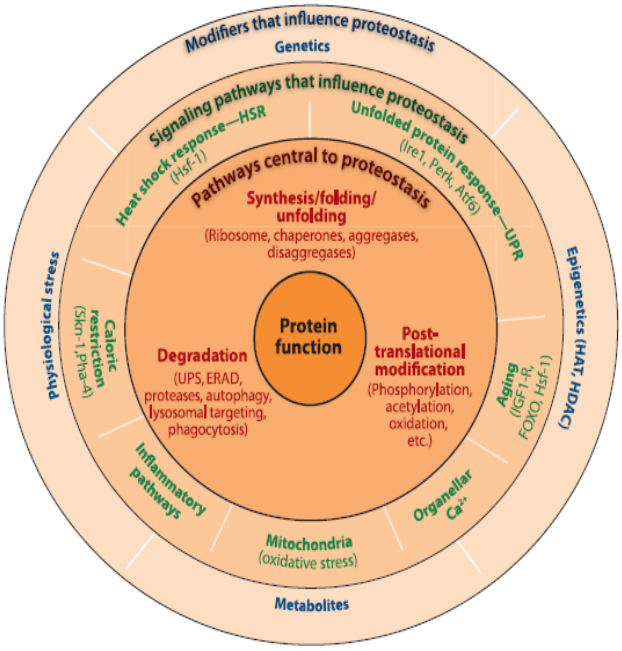

Fig. 15 managing proteostasis. The proteostasis network is composed of the components outlined in the first layer (red font), including the ribosomes, chaperones, aggregates and disaggregates that influence folding, as well as pathways that select proteins for degradation [the ubiquitin-proteasome system (UPS), endoplasmic reticulum-associated degradation (ERAD) system, proteases, autophagic pathways, lysosomal/endosomal pathways and phagocytic pathways, the latter responsible for the recognition, uptake and degradation of extracellular proteins]. The second layer includes signaling pathways (green font) that influence the activity of component found in the first layer. The third layer (blue font) includes genetic and epigenetic pathways, physiological stressors, and intracellular metabolites that affect the activity defined by the first and second layers. From: Powers et al Annu. Rev. Biochem. 2009

When a protein fails to fold properly owing to an alteration in its sequence (a DNA sequence mutation), an aberrant posttranslational modification, or when a misfolding-prone protein is overexpressed, or the composition of the components of the proteostasis network becomes deficient, cells have to face stressful conditions. Loss of protein function, or gain of toxic function (the latter often being associated with aggregation) can trigger disease by interfering with this cellular function. The multiple role of the proteostasis network in maintaining the proteome and, therefore, normal physiology in response to challenges during development, aging and stress are only beginning to be appreciated.

To better understand how the protein folding system and the proteostasis network interact and influence the folding efficiency of a given protein, mathematical models have been proposed wherein protein folding energetic and proteostasis network components and concentrations are the variable (Powers et al., 2009). Furthermore, the concept of proteostasis boundary, that is the folding energetic required to achieve folding of a protein at a given proteostasis network capacity, has been introduced to illustrate what can go wrong in proteostasis diseases. Thus the detailed knowledge of the proteostasis of the endoplasmic reticulum and the activation of the unfolded protein response pathway *in vivo* are important in order to progress toward translating these insights into meaningful improvements in the diagnosis and treatments of many diseases associated with protein misfolding

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8. APPENDIX

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Identification and Functional Requirement of Cu(I) and Its Ligands within Coagulation Factor VIII*

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Luigina Tagliavacca‡, Namdoo Moon§, William R. Dunham§, and Randal J. Kaufman‡¶‖

From the ‡Department of Biological Chemistry and ¶Howard Hughes Medical Institute, University of Michigan Medical Center, and the §Biophysics Research Division, University of Michigan, Ann Arbor, Michigan 48109

Coagulation factor VIII (FVIII) is a heterodimer consisting of a light chain of 80 kDa (domains A3-C1-C2) in a metal ion-dependent association with a 220-kDa heavy chain (domains A1-A2-B). The nature of the metal ion-dependent association between the heavy and light chains was investigated using atomic absorption spectroscopy, electron paramagnetic resonance spectroscopy (EPR), and site-directed mutagenesis and expression of the FVIII cDNA. Whereas copper ion was not detected in intact recombinant FVIII, EDTA dissociation of the chains yielded an EPR signal consistent with 1 mol of Cu(I)/mol of active protein, supporting the hypothesis that a single molecule of reduced copper ion is buried within intact FVIII and is released and oxidized upon treatment with EDTA. Cu(I), and not Cu(II), was able to reconstitute FVIII activity from dissociated chains, demonstrating a requirement for Cu(I) in FVIII function. Three potential copper ion binding sites exist within FVIII: one type-2 site and two type-1 sites. The importance of these potential copper ion ligands was tested by studying the effect of site-directed mutants. Of the two histidines that compose the type-2 binding site, the His-1957 → Ala mutant displayed secretion, light and heavy chain assembly, and activity similar to wild-type FVIII, while mutant His-99 → Ala was partially defective for secretion and had low levels of heavy and light chain association and activity. In contrast, FVIII having the mutation Cys-310 → Ser within the type-1 copper binding site in the A1 domain was inactive and partially defective for secretion from the cell, and the heavy and light chains of the secreted protein were not associated. Mutant Cys-2000 → Ser within the A3 domain displayed secretion, assembly, and activity similar to that for wild-type FVIII. These results support the hypothesis that Cu(I) is buried within the type-1 copper binding site within the A1 domain and is required for FVIII chain association and activity.

Factor VIII (FVIII)[1] is the X-chromosome-linked gene product that is deficient or defective in the bleeding disorder hemophilia A. FVIII functions in the blood-clotting cascade as the cofactor for factor IXa proteolytic activation of factor X. FVIII has a domain organization of A1-A2-B-A3-C1-C2 and is synthesized as a single chain polypeptide of 2351 amino acids, from which a 19-amino acid signal peptide is cleaved upon translocation into the lumen of the endoplasmic reticulum (ER) (1, 2). Upon secretion from the cell, FVIII is further processed by cleavage after residue 1648 to yield a heterodimer consisting of a 220-kDa amino-terminal-derived heavy chain (domains A1-A2-B) associated in a metal ion-dependent manner with a carboxyl-terminal-derived light chain (domains A3-C1-C2) (3).

The FVIII heterodimer circulates in plasma in a complex with von Willebrand factor (vWF) in an inactive form that requires proteolytic cleavage by thrombin or factor Xa for release from vWF and generation of coagulant activity. Cleavage within the heavy chain after Arg-740 generates a 90-kDa fragment that is further cleaved after Arg-372 to yield fragments of 50 and 43 kDa (4, 5). The 80-kDa light chain is cleaved after Arg-1689 to generate a 73-kDa polypeptide. Activated FVIII (FVIIIa) is a heterotrimer of A1, A2, and A3/C1/C2 subunits. The A1 and A3/C1/C2 subunits interact through a stable metal ion-dependent association between the A1 and A3 domains, while the A2 domain is associated with the A1 domain through a weak electrostatic interaction (6).

The triplicated A domains of FVIII exhibit 35–40% amino acid identity to each other and to the triplicated A domains in coagulation factor V (FV) and in the copper-binding protein ceruloplasmin (7–9). Each of the A domains exhibits conserved disulfide bridges and can be subdivided into two subdomains (subdomains d1–d6 for the three A domains in FVIII, FV, and ceruloplasmin). Each d subdomain resembles the β-barrel folded structure of the ancient copper ion-binding protein cupredoxin (10).

The blue copper-containing proteins and proteins related to them form a heterogeneous group that extend from the small blue proteins in bacteria and plants, to the blue oxidases in plants and mitochondria, and to the coagulation factors V and VIII in vertebrate plasma (11). These proteins contain copper ions of three types that are defined by the amino acid ligands that constitute the binding site. The blue copper-containing proteins can bind from 1 to 9 copper atoms, where the blue color is attributed to the presence of a type-1 copper binding site with characteristic spectral and redox properties (12). The blue oxidases and related proteins, which include the plant proteins laccase, ascorbate oxidase, and the plasma protein ceruloplasmin, contain an intensely blue type-1 Cu(II) ion with absorption in the visible region, a trinuclear cluster consisting of a pair of magnetically coupled type-3 copper ions characterized by a strong absorption in the near-ultraviolet region and the absence of an EPR signal, and a magnetically isolated type-2 Cu(II) ion having an undetectable absorption. The type-1 and type-2 Cu(II) ions have unique EPR spectra that disappear and absorption properties that change upon reduction of the copper ion to Cu(I) (13). Laccase and ascorbate oxidase perform oxida-

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‖ To whom correspondence should be addressed. Tel.: 313-763-9037; Fax: 313-763-9323; E-mail: kaufmanr@umich.edu.

[1] The abbreviations used are: FVIII, factor VIII; FV, factor V; vWF, von Willebrand factor; EPR, electron paramagnetic resonance; WT, wild-type; PAGE, polyacrylamide gel electrophoresis; ALLN, *N*-α-Leu-Leu-norleucinal; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay.

tion reactions by transferring four electrons from a reducing substrate to a molecule of oxygen that is subsequently reduced to water. Sequence alignments and three-dimensional structures support the hypotheses that the small blue proteins and the blue oxidases are homologous (14) and evolved from a common origin through gene duplication and divergence (11, 15). The evolutionary and structural conservation of the A domains between the coagulation factors and the blue oxidases suggests a role for copper ion binding in the coagulation factors. Indeed, approximately 1 mol of copper ion/mol of protein was detected directly in FVIII by atomic absorption spectroscopy (16) and in FV by atomic absorption and atomic emission spectroscopy (17). At present, there is no information regarding the oxidation state or the ligands that coordinate copper ion in FVIII or FV.

Based on the homologies to ancient copper-binding proteins and the known structures for the ancient copper-binding proteins, it is hypothesized that copper ion may be important for the metal ion-dependent interaction between the A1 and A3 domains of FVIII. The nature of the metal ion-dependent bridge between the FVIII heavy and light chains was investigated previously using different strategies. Incubation of the plasma-derived FVIII with a chelating agent, such as EDTA, dissociated the heavy and light chains and destroyed coagulant activity (18). Reconstitution of the FVIII heavy and light chain subunits with different divalent cations showed specific ion requirements and ionic strength dependence. Under these *in vitro* conditions, Mn(II) was the most effective cation in regenerating FVIII activity, yielding 30% reconstitution, whereas Ca(II) yielded 18% reconstitution. In contrast, copper ion, in the form of cupric solution, did not promote reconstitution (19, 20). Atomic absorption spectrometry analysis of FVIII heavy and light chain heterodimers and thrombin-activated FVIII complexes detected copper ion only when the chains were associated (16). In contrast, neither magnesium or manganese were detected.

At present it is unknown what amino acids within FVIII are responsible for coordinating the copper ion. The predicted amino acid sequence of FVIII contains two consensus type-1 copper ion binding sites, each defined by the canonical ligands His-Cys-His-Met that are located in the A1 and A3 domains, respectively (15). However, to date, no direct experimental evidence supports the hypothesis that these potential copper binding sites are occupied by copper ion. An initial structural prediction of the arrangement of the FVIII A domains was made based on the homology with the copper ion-binding protein nitrite reductase (21). More recently, this model was refined based on the 3-Å structure of ceruloplasmin (22, 23). In these models, a type-2 copper ion binding site composed of histidine residues at positions 99 and 1957 is proposed to bridge the A1 and A3 domains of FVIII. To elucidate the nature of the copper ion binding site in FVIII, we performed chemical analysis, functional assays, and site-directed mutagenesis to identify the copper ion content and its ligands within FVIII.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant FVIII, anti-heavy chain factor VIII monoclonal antibody (F-8), and F-8 conjugated to CL-4B Sepharose were obtained from Genetics Institute Inc. (Cambridge, MA). Centriplus columns were purchased from Amicon Inc. (Beverly, MA). Light chain factor VIII monoclonal antibodies ESH-4 and ESH-8 were purchased from American Diagnostica Inc. (Greenwich, CT). Coamatic chromogenic factor VIII activity assay kit was purchased from Pharmacia Hepar (Franklin, OH). Factor VIII-deficient plasma was obtained from George B. King Biomedical Inc. (Overland Park, KS). Activated partial thromboplastin reagent and $CaCl_2$ were purchased from General Diagnostics Organ Technique Corp. (Durham, NC). Human α-thrombin was from Hemtological Technology Inc. (Burlington VT). Soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, and aprotinin were purchased from Boehringer Mannheim. *N*-α-Leu-Leu-norleucinal (ALLN) was purchased from Sigma. [^{35}S]Methionine (>1000 Ci/mmol) was purchased from Amersham Life Science Inc. EN^{3}HANCE was purchased from DuPont Corp. Dulbecco's modified Eagle's medium and methionine-free Dulbecco's modified Eagle's medium were obtained from Life Technologies, Inc. Chelex-100 resin was purchased from Bio-Rad.

Atomic Absorption Analysis—Atomic absorption measurements were made on a Perkin Elmer 3300. Recombinant FVIII protein (350 μl) was diluted 1/1 in 1% HNO_3 (by volume), injected in the pyrolitic graphite tube heated by joule effect with the following program: 120 K for 50 s, 1000 K for 30 s, and 2300 K for 5 s. The calibration curve was obtained by dilution of the standard copper solution in 0.5% HNO_3. Each value was the average of two measurements.

Spectroscopy—EPR measurements were made using a Varian Century line X-band (9 GHz) EPR spectrometer equipped with a cryogenic Dewar system. The conditions for the detection of the cupric ions were as follows: microwave power, 10 milliwatts; microwave frequency, 9.17 GHz; modulation frequency, 100 kHz; amplitude, 0.1 millitesla; temperature, 130K. For each spectrum, the analog output was recorded digitally on the computer via a data acquisition board made by ComputerBoard Inc. (Mansfield, MA).

Activation of FVIII—Human recombinant FVIII in 50 mM Tris-HCl (pH 7.2), 0.4 mol/liter NaCl, 5 mM $CaCl_2$, and 0.1% Tween 80 was incubated at 37 °C with human α-thrombin. At short intervals, aliquots were removed and assayed for FVIII clotting activity by the one-stage clotting assay using FVIII-deficient plasma.

Dissociation of FVIII Subunits and Reconstitution of FVIII Activity—Highly purified recombinant FVIII was treated with 50 mM EDTA, pH 8.0, for 16 h at room temperature. The rate of dissociation was monitored by loss of activity measured by the one-stage clotting assay. The reaction was dialyzed against a metal ion-free buffer containing 50 mM Tris, pH 7.5, 0.15 M NaCl, 2.5 mM $CaCl_2$, 5% glycerol. Metal-free water, filtered through a Chelex-100 resin, was used throughout the experiment. The reconstitution of FVIII activity was performed by adding solutions of cupric chloride or cuprous chloride to the dissociated FVIII protein (35 μg/ml) as indicated. Reactions were performed at room temperature, and aliquots were removed at the indicated times for measure of activity using the one-stage clotting assay.

Plasmid Mutagenesis—The FVIII expression vector pMT2-VIII MluI-1648 has been described previously (24). Prior studies demonstrated that the introduction of the *Mlu*I restriction site (yielding a Thr-Arg at amino acid residues 1647–1648) did not alter the synthesis, secretion, or functional activity of the molecule. Site-directed oligonucleotide-mediated mutagenesis was performed by polymerase chain reaction procedures (25). Codon 99 was mutated from CAT to GCT predicting an amino acid change from histidine to alanine (His-99 → Ala), codon 310 was mutated from TGT to AGT predicting an amino acid change from cysteine to serine (Cys-310 → Ser), codon 1957 was mutated from CAT to GCT predicting an amino acid change from histidine to alanine (His-1957 → Ala), and codon 2000 was mutated from TGC to TCC predicting an amino acid change from cysteine to serine (Cys-2000 → Ser). All mutations were confirmed by DNA sequencing over the mutagenized region and extensive mapping with restriction endonuclease enzymes.

DNA Transfection and Analysis—Plasmid DNA was transfected into COS-1 monkey cells by the diethylaminoethyl (DEAE)-dextran procedure (26). Conditioned medium was harvested 60 h after transfection in the presence of 10% heat-inactivated fetal bovine serum for FVIII assay. Primary translation products were analyzed by pulse-labeling cells for 30 min with [^{35}S]methionine (250 μCi/ml in methionine-free medium) and preparing cell extracts in a Nonidet P-40 lysis buffer (27). Protein secretion was monitored by metabolic pulse-labeling cells for 30 min with [^{35}S]methionine (250 μCi/ml) and chasing for the indicated period of time in medium containing an excess of unlabeled methionine as described (28). The FVIII was immunoprecipitated from the cell extract and conditioned medium with an anti-heavy chain factor VIII monoclonal antibody F-8 (27) coupled to CL-4B Sepharose. The immunoprecipitates were washed as described (27) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a low bisacrylamide containing 8% polyacrylamide gel. Proteins were visualized by autoradiography after fluography by treatment with EN^{3}HANCE. The band intensities were quantitated by scanning the lanes using an LKB UltroScan XL laser densitometer (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Chain association was measured by co-immunoprecipitation experiments. After immunoprecipitation with an anti-heavy chain antibody F-8, the supernatant was subsequently immunoprecipitated with the anti-light chain monoclonal antibody ESH-4 coupled to Affi-Gel. Immu-

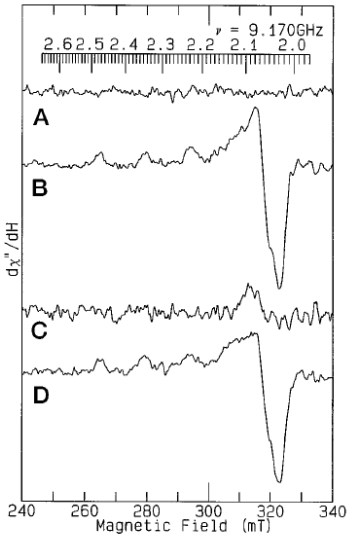

FIG. 1. **EPR spectra of FVIII.** EPR spectra were obtained as described under "Experimental Procedures." *A*, recombinant FVIII in 50 mM Tris-HCl, pH 7.2, 0.4 M NaCl, 2.5 mM $CaCl_2$, 0.1% Tween 80; *B*, FVIII incubated in 50 mM EDTA; *C*, FVIII digested with human thrombin; *D*, FVIII digested with thrombin and subsequently incubated with 50 mM EDTA.

noprecipitated proteins were analyzed by SDS-PAGE as described above.

FVIII Assay—Factor VIII activity was measured by the coamatic chromogenic assay (Coamate) according to the manufacturer or by the one-stage activated partial thromboplastin time clotting assay using FVIII-deficient plasma. The antigen level was measured by an enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibodies ESH-4 and ESH-8 that recognize the FVIII light chain (29). For highly purified FVIII, the protein concentration was measured by the Bradford method (30).

RESULTS

Atomic Absorption and EPR Detection of Copper Ion in FVIII—Homogeneous recombinant FVIII, purified from Chinese hamster ovary cells was analyzed by atomic absorption to determine the copper ion content and to correlate the amount of copper ion/mol of active protein. The specific activity of the recombinant FVIII was 1250 units/mg, as measured by the one-stage clotting assay and Bradford protein assay. Atomic absorption spectroscopy detected 484 nM copper ion/500 nM active protein, consistent with 1 mol of copper ion/mol of active FVIII protein, and is consistent with a previous report (16). For EPR analysis, FVIII was concentrated through a Centriplus column and the antigen was measured by ELISA using anti-light chain antibodies. The concentrated protein (55 μM) was analyzed by EPR to determine the copper ion content. EPR spectroscopy of frozen FVIII solution revealed no EPR detectable copper ion (Fig. 1*A*). In addition, the presence of other paramagnetic metal ions, including Mn^{2+}, was not detected. Since atomic absorption spectrometry identified 1 mol of copper ion/mol of active FVIII protein and copper ion would be silent by EPR if it were protected within the protein in a reduced form, we tested whether heavy and light chain dissociation by metal ion chelation would release and oxidize protected copper ion. The FVIII heavy and light chains were separated by incubation with EDTA as previously reported (18), and the dissociation was monitored by inactivation of FVIII activity measured in the one-stage clotting assay. After overnight incubation with 60 mM EDTA, the amount of coagulant FVIII activity decreased to less than 1% of the initial activity. EPR spectroscopy of the dissociated FVIII detected a significant copper ion signal corresponding to 17 μM, supposedly as an EDTA-chelated complex (Fig. 1*B*). EPR analysis of the EDTA solution excluded contamination of copper ion. In addition, an EPR signal for copper ion was not detected in the cavity. These data demonstrate that native recombinant FVIII contains copper ion in a reduced form Cu(I) that is EPR silent. EDTA treatment dissociated the FVIII heavy and light chains, destroyed coagulant activity, and fully oxidized Cu(I) to Cu(II), thus allowing the recovery and detection of copper ion in the oxidized form.

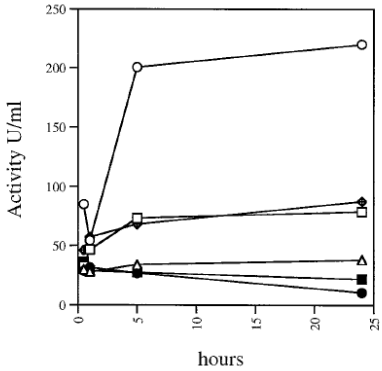

FIG. 2. ***In vitro* reconstitution of FVIII activity.** Purified EDTA-dissociated FVIII at 35 μg/ml was incubated at room temperature alone (△), or in the presence of 10 μM Cu(I) (○), 100 μM Cu(I) (●), 10 μM Cu (II) (□), 100 μM Cu(II) (■), or 10 μM Cu(I)-GSH (◆). Portions of the reactions were taken at 1.5 and 24 h for FVIII activity determination by the one-stage clotting assay.

We next tested whether thrombin cleavage and activation may release bound copper ion. After incubation with thrombin, we observed a rapid increase in the coagulant activity, followed by a first-order decay in activity that is known to be accompanied by dissociation of the A2 domain subunit (31, 32). EPR analysis was performed 3 h after thrombin cleavage, at a time when most of FVIII activity had decayed. The analysis did not detect copper ion (Fig. 1*C*). Incubation of the thrombin-treated sample with 50 mM EDTA for 30 min promptly yielded a detectable Cu-EDTA complex (Fig. 1*D*). These results demonstrate that thrombin cleavage and activation are not accompanied by oxidation of bound copper ion and that thrombin-activated FVIII still retained Cu(I) in an EDTA-releasable state. The observation that Cu(I) is present in native FVIII provided the rationale to test whether Cu(I) could promote *in vitro* reconstitution of FVIII from dissociated heavy and light chains.

Cu(I), but Not Cu(II), Promotes Reconstitution of FVIII Activity—Reconstitution of FVIII activity from EDTA-dissociated subunits was performed using different copper ion containing solutions in the reduced or oxidized forms. Cuprous chloride was most effective in promoting FVIII activity (Fig. 2). Complete reconstitution (where the reconstituted FVIII exhibited the same specific activity as the starting material) occurred with 10 μM Cu(I), which represents an approximate ratio of 30–50 mol of Cu(I)/mol of FVIII. Cuprous chloride at 100 μM inhibited reconstitution of FVIII activity, probably due to nonspecific binding of the metal ion to the subunits. The reconsti-

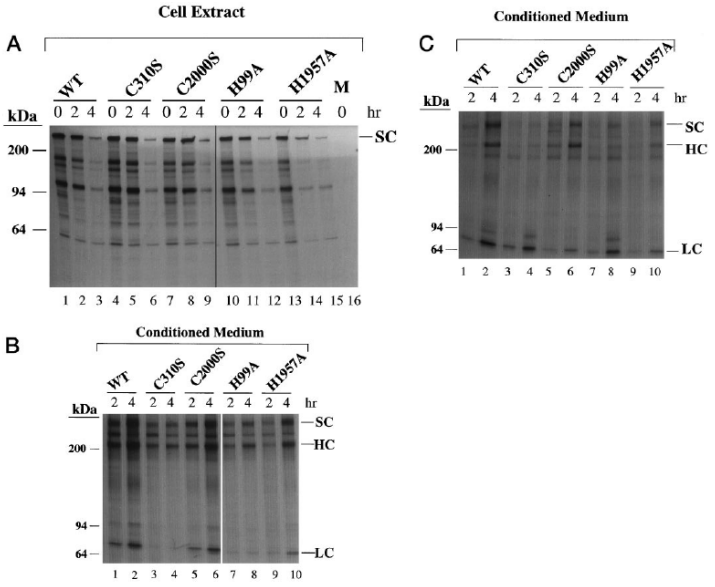

FIG. 3. **Expression of FVIII WT and mutants in COS-1 cells.** WT and mutant FVIII expression plasmids were transfected into COS-1 monkey cells. At 60 h post-transfection, cells were pulse-labeled with [^{35}S]methionine for 30 min and the cell extracts were harvested and immunoprecipitated with anti-FVIII heavy chain antibody (*A*). Duplicate plates were labeled for 30 min and chased for 2 h or 4 h in medium containing excess unlabeled methionine, and then cell extracts and conditioned medium were harvested. Equal volumes were immunoprecipitated with anti-FVIII heavy chain antibody and were analyzed by SDS-PAGE (*B*). The supernatants from the conditioned medium samples were subsequently immunoprecipitated with an anti FVIII light-chain antibody (*C*). The migration of the FVIII in the cell extract is detected as a single chain (*SC*). FVIII in the conditioned medium is detected as single chain (*SC*), heavy chain (*HC*), and light chain (*LC*) species. *M* indicates mock cells that did not receive any plasmid DNA.

tution of FVIII activity reached a plateau after 5 h at room temperature and maintained the activity for at least 24 h at room temperature indicating a stable interaction and assembly of the heavy and light chain subunits in the presence of reduced copper ion. By contrast, the reconstitution of FVIII activity from dissociated heavy and light chain subunits in the absence of metal ion (control) gave only background activity. Cupric solutions, at the same molar ratio as the cuprous solutions, were evaluated for their ability to promote reconstitution. A solution of 10 μM Cu(II) yielded a 2-fold increase of FVIII activity, while 100 μM Cu(II) was ineffective.

Glutathione (GSH) is the most abundant ligand for copper ion within the mammalian secretory pathway. *In vitro* reconstitution of the copper-containing proteins ceruloplasmin (33), Cu,Zn-superoxide dismutase (34), apohemocyanin (35), and thioneins (36) occurs by means of the copper ion associated in a glutathione complex as Cu(I)-GSH. Under our conditions of reconstitution, Cu(I)-GSH was poorly effective in reconstituting FVIII activity (Fig. 2).

Site-directed Mutagenesis at the Proposed Type-1 Copper Binding Sites—Because copper ion within FVIII is detectable only after chelation with EDTA, it is not possible to elucidate the coordination properties of copper ion in FVIII by EPR analysis. Therefore, we tested the significance of the potential copper ion ligands within FVIII by site-directed mutagenesis. FVIII contains two consensus type-1 copper ion binding sites that are located in the A1 domain (composed of residues His-265, Cys-310, His-315, and Met-320) and A3 domain (composed of residues His-1954, Cys-2000, His-2005, and Met-2010). Because the Cys residue is an essential component of the type-1 copper ion binding site (37), we mutated each of the two Cys residues Cys-310 and Cys-2000 to the conserved residue Ser to determine their requirement in FVIII chain association and activity. Expression vectors containing the wild-type FVIII cDNA or mutants bearing either of the Cys to Ser substitutions were transfected into COS-1 monkey cells. At 60 h post-transfection, FVIII synthesis and secretion were evaluated by [^{35}S]methionine metabolic pulse labeling and chase analysis. Samples of cell extracts and conditioned medium were analyzed by immunoprecipitation with anti-FVIII heavy chain antibody and SDS-PAGE. Analysis of the cell extracts demonstrated that wild-type FVIII and the mutants were synthesized as single chain polypeptides migrating at approximately 280 kDa (Fig. 3*A*). Quantitation of the band intensities demonstrated similar amounts of radiolabel incorporation into FVIII protein for all the constructs indicating similar translation rates. In addition, analysis of the 2- and 4-h chase time points demonstrated that the wild-type and both mutants disappeared from the cell extract at similar rates, suggesting similar rates of intracellular degradation and/or secretion.

The FVIII secreted into the conditioned medium over the 2–4-h chase period was collected and subjected to immunoprecipitation analysis. FVIII chain association was analyzed by immunoprecipitation of the conditioned medium with anti-FVIII heavy chain antibody. The supernatant was subsequently immunoprecipitated with anti-FVIII light chain anti-

TABLE I
Specific activity of FVIII WT and mutants secreted into conditioned medium

Plasmid DNA was transfected into COS-1 cells. The cells were fed fresh medium after 40 h. After 24 h the conditioned medium was collected for FVIII activity analysis by the chromogenic assay and ELISA for quantitation of antigen. Cells were then labeled with [^{35}S] methionine and analyzed as described in Fig. 3. The activity data are the average of three independent transfection experiments.

Plasmid DNA	Coatest assay	Antigen	Specific activity
	% WT ± S.D.	*% WT ± S.D.*	*% WT*
WT	100	100	100
Cys-310 → Ser	1.2 ± 3.4	72 ± 1.5	2.2
Cys-2000 → Ser	51 ± 19	35 ± 7.7	128
His-99 → Ala	19 ± 3.3	78 ± 6.1	27
His-1957 → Ala	68 ± 40	60 ± 7.7	122

body. Since the anti-heavy chain antibody does not recognize the light chain and the anti-light chain antibody does not recognize the heavy chain, analysis of the amount of co-immunoprecipitation indicates the extent of chain association. After immunoprecipitation with the anti-heavy chain antibody, wild-type FVIII is detected in the conditioned medium as a single chain of 300 kDa, a heavy chain of 220 kDa, and an associated light chain of 80 kDa. FVIII wild-type and mutants appeared in the conditioned medium after the 2-h chase time point, although the amount of single chain and heavy chain detected for the Cys-310 → Ser mutant were slightly less than the respective species for the wild-type protein (73% of wild-type) (Fig. 3*B*). The amount of heavy chain for wild-type and Cys-2000 → Ser mutant FVIII increased 1.5-fold between the 2-h and 4-h chase time points. In contrast, the amount of Cys-310 → Ser mutant secreted into the conditioned medium did not significantly increase during this period. After the 4-h chase time point, the amount of the secreted heavy chain for the Cys-310 → Ser mutant was about 50% of the wild-type. Significantly, compared with wild-type, the heavy and light chain were not associated for the Cys-310 → Ser mutant, as a very low level of light chain was co-immunoprecipitated with the anti-heavy chain antibody (Fig. 3*B*, compare *lanes 1* and *2* with *lanes 3* and *4*).

Immunoprecipitation with the anti-light chain FVIII antibody demonstrated that the amount of light chain present in the conditioned medium at the 2- and 4-h chase time points for the Cys-310 → Ser mutant was similar to the wild-type (Fig. 3*C*; compare *lanes 1* and *2* with *lanes 3* and *4*). In the wild-type FVIII, there was a considerable amount of heavy chain that co-immunoprecipitated with the anti-light chain antibody. This again indicates a complex between the heavy chain and the light chain and that the initial immunoprecipitation with anti-heavy chain antibody was not quantitative under these conditions. In contrast, heavy chain was not detected upon immunoprecipitation of the Cys-310 → Ser mutant with the anti-light chain antibody, supporting the absence of a heavy and light chain complex. Analysis of the FVIII activity in the conditioned medium demonstrated that activity for the Cys-310 → Ser mutant was at least 100-fold reduced compared with the wild-type or the Cys-2000 → Ser mutant and was not detectable above the background (Table I). These results show that the Cys-310 → Ser mutant was properly synthesized, transported from the ER to the Golgi compartment where the protein was cleaved to its mature heavy and light chains, and secreted as dissociated chains. However, the heavy chain did not accumulate in the conditioned medium. At present, we do not know if the heavy and light chain are unassociated in the Golgi compartment or are rapidly dissociated upon secretion into the conditioned medium.

In contrast to the Cys-310 → Ser mutant, the Cys-2000 → Ser mutant displayed a secretion rate similar to wild-type, and the heavy and light chains were associated as detected by co-immunoprecipitation with the anti-FVIII heavy chain antibody (Fig. 3*B*, *lanes 5* and *6*). The activity of the secreted protein was 50% of wild-type FVIII, while the specific activity was actually higher than the wild-type. The higher specific activity may be due to a conformational change in the epitope recognized by the light chain antibodies used to measure the antigen level by ELISA (Table I). This is supported by the observation that immunoprecipitation with anti-heavy chain antibody detected similar amounts of protein for the Cys-2000 → Ser mutant and wild-type. Thus, similar amounts of FVIII were detected by the immunoprecipitation analysis, whereas the ELISA using anti-light chain antibodies detected less of the Cys-2000 → Ser mutant.

Site-directed Mutagenesis at the Proposed Type-2 Copper Binding Site—A structural model of the FVIII A domains based on the crystal structure of ceruloplasmin predicts a type-2 copper ion binding site that coordinates the interaction between the A1 and A3 domains. The two histidines proposed to coordinate the copper ion are His-99 within the A1 domain and His-1957 within the A3 domain (21). Expression of FVIII with the His-1957 → Ala substitution in COS-1 cells produced 60% activity of the wild-type and proportionally a lesser amount of protein secreted into the conditioned medium. Immunoprecipitation of the conditioned medium with either the anti-heavy or anti-light chain antibodies demonstrated that the heavy and light chain for the His-1957 → Ala mutant were associated to a similar extent as wild-type (Fig. 3*B*, *lanes 9* and *10*). These results indicate that mutation of His-1957 → Ala results in a FVIII protein that has its heavy chain associated with the light chain. The reduced activity for the His-1957 → Ala mutant in the conditioned medium is attributable to less efficient secretion. However, as for the Cys-2000 → Ser mutant, the specific activity of the His-1957 → Ala mutant was higher than wild-type (Table I), and possibly reflects weaker interaction with the antibody used in the ELISA. These results indicate that His-1957 does not play an essential role in heavy and light chain association and/or cofactor activity.

We also studied mutation of His-99 within the A1 domain. The His-99 → Ala mutant yielded a FVIII protein that was synthesized at a rate similar to wild-type FVIII (Fig. 3*A*, *lanes 10–12*). Although the protein disappeared from the cell extract at a rate similar to wild-type, it was less efficiently recovered in the conditioned medium (Fig. 3*B*, *lanes 7* and *8*). Immunoprecipitation with anti-heavy chain antibody demonstrated that the His-99 → Ala heavy chain was less efficiently secreted than wild-type (70% of the wild-type) and displayed less light chain association. The activity of the secreted protein was approximately 20% of the wild-type (Table I).

To quantitate the extent of chain association for the Cys-310 → Ser and His-99 → Ala mutants that were poorly secreted and difficult to detect, we immunoprecipitated equal numbers of counts of conditioned medium with the anti-heavy chain antibody and subjected the samples to SDS-PAGE. PhosphoImage quantitation demonstrated that, compared with wild-type, the Cys-310 → Ser mutant displayed 7% chain association and the His-99 → Ala mutant displayed 16% chain association. The results show that the Cys-310 → Ser mutant is severely defective in chain association while the His-99 → Ala mutant is partially defective in chain association.

Cys-310 → Ser and His-99 → Ala FVIII Are Not Targeted to Intracellular Degradation—The mutants Cys-310 → Ser and His-99 → Ala mutant FVIII molecules were properly synthesized and chased out of the cell but were poorly recovered in the conditioned medium. To investigate whether intracellular deg-

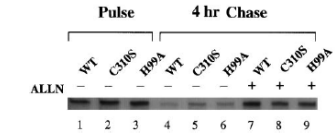

FIG. 4. **Cys-310 → Ser and His-99 → Ala FVIII mutants do not preferentially accumulate within the cell in the presence of ALLN.** WT and mutant FVIII expression vectors were transfected into COS-1 monkey cells. At 60 h post-transfection, the cells were labeled with [^{35}S]methionine for 30 min and chased for 4 h in the absence (*lanes 4–6*) or in the presence (*lanes 7–9*) of 250 μM of ALLN. The cell extracts were harvested, and equal volumes were immunoprecipitated with anti-FVIII heavy chain antibody for analysis by SDS-PAGE. Bands represent the single chain form of FVIII detected in the cell extracts.

radation was responsible for the reduced recovery of these mutants in the conditioned medium, we studied the effect of intracellular protease inhibition. ALLN is a cysteine protease inhibitor that prevents the degradation of FVIII retained within the secretory pathway (38). At 60 h post-transfection, cells were metabolically pulse-labeled for 30 min and chased for 4 h in medium containing ALLN. Equal amounts of cell extract and conditioned medium were collected, immunoprecipitated with the anti-FVIII heavy chain specific antibody, and analyzed by SDS-PAGE. Treatment with ALLN showed a similar increase in FVIII in the cell extract for the wild-type FVIII as for the mutant FVIII (Fig. 4). In addition, there was no increase in FVIII protein detected in the conditioned medium (data not shown). These results show that inhibition of intracellular degradation did not promote accumulation of mutant protein in the cell extract. In addition, it was not possible to rescue the secretion of either the Cys-310 → Ser or the His-99 → Ala mutant FVIII into the conditioned medium by cysteine protease inhibition. We conclude that the reduced amount of Cys-310 → Ser and His-99 → Ala FVIII protein detected in the conditioned medium is probably not due to degradation within the ER.

DISCUSSION

A structural model of the A domains of FVIII would be helpful in understanding its interaction with the other proteins in the coagulation cascade. The large size, heterogeneity, and extensive post-translational modifications of FVIII make it difficult to obtain a crystallographic structure. Most of the present information concerning FVIII structure are obtained from the interpretation of results from *in vitro* transfection studies of the wild-type or mutant FVIII molecules bearing specific amino acid changes. Although computer modeling of FVIII based on homology with the copper-binding proteins nitrite reductase (21) or ceruloplasmin (23) was attempted, there is no direct experimental evidence to support models for the interaction of the FVIII A1 and A3 domains.

Of critical importance to understanding FVIII structure is the question of where and what nature is the metal ion bridge that tethers the A1 domain to the A3 domain. Our atomic absorption spectroscopy demonstrated that recombinant FVIII contained copper ion at 1 mol/mol of active protein. EPR analysis detected copper ion in FVIII only after EDTA-induced release and oxidation, supporting that Cu(I) is buried within the molecule and protected from the oxidizing environment. EPR analysis did not detect copper ion in thrombin-inactivated FVIII, unless it was also EDTA-treated, suggesting that copper ion is bound and protected within the A1-A3/C1/C2 heterodimer. The detection of EDTA-releasable Cu(I) within FVIII before and after thrombin cleavage, indicates that FVIII activation is not associated with Cu(I) oxidation and excludes an electron transfer function for the copper ion in the activation process. Taken together, the results suggest that the metal ion within FVIII provides primarily a structural role. Finally, cuprous chloride, and not cupric chloride, promoted *in vitro* reconstitution of FVIII activity from dissociated heavy and light chain subunits, confirming the Cu(I) requirement for FVIII function.

Amino acid sequence analysis of FVIII predicts two type-1 mononuclear copper binding sites in the A1 and A3 domains that are conserved as free cysteines in the plasma copper-binding protein ceruloplasmin, where they coordinate a mononuclear copper ion with redox function (22). Our mutagenesis experiments support the importance of Cys-310 within the A1 domain in coordinating the type-1 copper binding site and rule out the importance of the type-1 copper binding site within the A3 domain of FVIII.

By computer modeling, His-1957 and His-99 were proposed to form a pocket of positive charge capable of coordinating a type-2 copper ion molecule (23). However, mutation of His-1957 to Ala did not affect the folding, secretion, or activity of the molecule, indicating either that the His ligand is substituted by a molecule of water in that mutant or that His-1957 does not coordinate copper ion. FVIII with His-99 mutated to Ala exhibited a moderate effect on activity and did not completely impair chain association of the secreted protein. Analysis of FVIII protein by means of the fluorescent, apolar probe bisanilinonaphthalsulfonic acid (bis-ANS), that specifically binds to hydrophobic sites detected two regions within the A1 and A3 domains that are exposed only after dissociation of the A1-A3/C1/C2 heterodimer (39). One of these regions contains nonpolar residues adjacent to His-99. Our mutagenesis data support a positive role for His-99 in promoting chain association. We propose that Cys-310 is an essential component of the type-1 copper binding site in the A1 domain that coordinates Cu(I) and provides an essential role for A1 domain folding so that the A1 domain can interact with the A3 domain, and that His-99 is also essential for the A1 and A3 domain interaction but is not a ligand for copper ion.

Our results support a requirement for Cu(I) interaction within FVIII for proper assembly of the heavy and light chains. Of crucial importance is where within the biosynthetic pathway the copper ion is required for FVIII assembly. It may be required within the ER, the Golgi compartment, or upon secretion of FVIII from the cell. The region adjacent to the proposed type-1 copper binding site within the A1 domain of FVIII contains a hydrophobic cluster of amino acids predicted to be a potential binding site for the ER protein chaperone BiP (40). Compared with FV, FVIII displays a reduced secretion efficiency that correlates with BiP interaction within the ER. In contrast, FV does not interact with BiP (41, 42). Exchange of residues containing this hydrophobic cluster can confer the secretion properties of FV onto FVIII, although the efficiently secreted molecule is not active, and the heavy and light chains are not associated (40). Within this cluster, Phe-309, adjacent to Cys-310, is the most responsible for BiP interaction and/or inefficient folding (43). FV does not have the typical sequence requirements for the proposed type-1 copper binding site, having Ser at the position corresponding to Cys-310 within FVIII (44). Substitution of Cys at position 310 with Ser in the A1 domain of FVIII generated a molecule in which the heavy and light chains were not associated and the protein was not active. These data provide strong support for the hypothesis that the copper ion in FVIII is liganded to different residues than the copper ion in FV. It is interesting that a potential high affinity BiP binding site is adjacent to the Cys-310 ligand in the type-1 copper ion binding site that is required for heavy and light

chain association. Bip interaction adjacent to Cys-310 may facilitate CU(1) binding, possibly by preventing oxidation of the cysteine residue. We propose that the different secretion efficiencies between FV and FVIII may be a consequence of their different requirements for copper ion binding within the A1 domain.

If the assembly of FVIII heavy and light chains with reduced copper ion occurs in the oxidizing environment of the secretory pathway, then there must be a mechanism to prevent oxidization of Cu(I) to Cu(II). This observation leads to the hypothesis that another protein may be involved in delivering Cu(I) to FVIII within the secretory pathway. Although the mechanism of copper transport and trafficking within the cell is poorly understood, at least two genes are known to be involved in copper homeostasis in humans. Defects in either of the two genes lead to Wilson's or Menkes' disease (45–48). The protein products of these genes reside in the Golgi compartment, are members of the P-type ATPase family, and are responsible for the translocation of copper ions across the intracellular membrane within the secretory pathway (49). While 95% of the patients with Wilson's disease have ceruloplasmin deficiency (50), there are no reports of FV/FVIII deficiency in Wilson's or Menkes' patients. Analysis of the plasma FVIII level in 10 patients affected by Wilson's disease and ceruloplasmin deficiency demonstrated their levels to be normal.[2] This indicates that, although similar, the three proteins exhibit different requirements for copper ion in protein folding within the secretory pathway.

It is also possible that assembly of the FVIII heavy and light chains with Cu(I) occurs upon secretion from the cell. Pulse-chase analysis of the secreted FVIII mutant Cys-310 → Ser demonstrated that the amount of light chain secreted into the conditioned medium was similar to the wild-type and remained stable during the time course. Although the amount of the Cys-310 → Ser heavy chain detected at 2 h of chase was similar to the wild-type, it did not accumulate during a 4-h chase. This suggests that the heavy chain is unstable in the absence of light chain association. Taken together, these experiments support the conclusion that the Cys-310 → Ser mutant is expressed and secreted with the same efficiency as wild-type FVIII, however the heavy and light chains are not associated and the heavy chain is unstable in the conditioned medium. This phenotype is reminiscent of the observation that FVIII synthesized in the absence of vWF in the conditioned medium is secreted as separate heavy and light chains, of which the heavy chain is subsequently degraded (3). In addition, vWF can promote *in vitro* reconstitution of FVIII activity from dissociated subunits (51). These observations suggest that vWF may promote the association of the FVIII heavy and light chains upon secretion from the cell. The extremely rich cysteine content of the D domains of vWF is similar to that of metallothioneins (52) which are copper-binding proteins. It is possible that vWF may promote FVIII heavy and light chain association by delivering a copper ion to FVIII upon secretion from the cell.

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Biosynthesis, assembly and secretion of coagulation factor VIII

Randal J. Kaufman, Steven W. Pipe, Luigina Tagliavacca, Manju Swaroop and Micheline Moussalli

Factor VIII is a large complex glycoprotein that is deficient in hemophilia A. It has a domain organization consisting of A1–A2–B–A3–C1–C2 where the B domain is a heavily glycosylated region that is dispensable for procoagulant activity. Factor VIII expression is 10- to 20-fold lower than the homologous coagulation factor V. Factor VIII expression is limited due to a low level of steady-state messenger RNA in the cytoplasm and inefficient transport of the primary translation product from the endoplasmic reticulum to the Golgi apparatus. Within the secretory pathway, factor VIII is processed to a heterodimer of the heavy chain (domains A1–A2–B) in a metal ion association with the light chain (domains A3–C1–C2). Upon secretion from the cell, von Willebrand factor binds the light chain of factor VIII and stabilizes the factor, preventing degradation. Protein folding within the mammalian secretory pathway is facilitated by molecular chaperones. Within the endoplasmic reticulum, factor VIII exhibits stable interaction with protein chaperones identified as the immunoglobulin-binding protein (BiP), calnexin and calreticulin. BiP is a peptide-dependent ATPase that interacts with exposed hydrophobic surfaces on unfolded proteins or unassembled protein subunits. A potential BiP binding site within factor VIII has been identified. Mutation of a single amino acid residue in the potential BiP binding site increased the secretion efficiency of factor VIII by threefold. Interestingly, the proposed BiP binding site is adjacent to a type-1 copper binding site within the A1 domain that is required for interaction between the factor VIII A1 domain and the A3 domain. We propose that Cu(I) binds the type-1 copper ion-binding site in the A1 domain and provides the essential requirement for a stable interaction between the heavy and light chains. Calnexin and calreticulin are transmembrane and lumenal proteins, respectively, localized to the endoplasmic reticulum, which associate transiently with many soluble and membrane glycoproteins during folding and subunit assembly. The calnexin and calreticulin interaction with factor VIII occurs primarily through amino-terminal linked oligosaccharides within the heavily glycosylated factor VIII B domain and this interaction appears to be required for factor VIII secretion. The findings suggest that factor VIII cycles through interactions with BiP, calnexin and calreticulin. Although the interaction with BiP does not appear to be required for factor VIII secretion, data suggest that the calnexin and/or calreticulin interaction is required for secretion. The observations suggest a unique requirement for carbohydrate processing and calnexin/calreticulin interaction that may limit the productive secretion of factor VIII and have implications for approaches towards somatic cell gene therapy for hemophilia A.

Keywords: calnexin, calreticulin, carbohydrate, endoplasmic reticulum, immunoglobulin binding protein

Introduction

Hemophilia A is an X-chromosome-linked bleeding disorder caused by a deficiency in coagulation factor VIII. Factor VIII serves as a cofactor for the factor IXa-mediated proteolytic activation of factor X within the intrinsic blood coagulation cascade. Subsequently, factor Xa acts in the presence of its cofactor, activated factor V, to convert prothombin to its active enzymatic form, thrombin. Factor V and factor VIII are homologous glycoproteins that have a conserved domain organization of A1–A2–B–A3–C1–C2 [1–4] (Fig. 1). The A domains of factor VIII share 40% amino acid identity with each other and to the A domains of factor V and to the copper-binding protein ceruloplasmin [5], suggesting a role in copper ion binding. Consistent with this, studies have shown that both factor V and factor VIII contain 1 mol of copper per mol of protein [6,7]. The factor VIII C domains also exhibit 40% identity with each other, and with the C domains of factor V, and share homology with proteins that bind negatively charged phospholipids [8], suggesting a role in phospholipid interaction. The B domains of both cofactors are encoded by single exons and do not share homology with each other or with any other presently known gene. However, both B domains do contain a large number of asparagine-linked oligosaccharides [9,10]. The amino acid sequences within the factor VIII B do-

The authors are with the Departments of Biological Chemistry and Pediatrics, Howard Hughes Medical Institute, University of Michigan School of Medicine, Ann Arbor, MI 48109, USA. Address correspondence to Dr Randal J. Kaufman at the above address.

mains have also extensively diverged between human, porcine, and mouse genes [2,11], and the factor V B-domain sequences have extensively diverged between the human and bovine genes [10,12]. The conservation of the large number of asparagine-linked glycosylation sites with divergence of primary amino acid sequence suggests that glycosylation may regulate either the expression or the activation of these molecules.

Factor VIII and factor V circulate in plasma as inactive precursors that are activated through limited proteolysis by either thrombin or activated factor X (Xa). The activated forms of factors VIII and V assemble with their respective substrates (factor X and prothombin) and enzymes (factors IXa and Xa) on a negatively charged phospholipid surface in the presence of calcium ions. Both cofactors act to increase the maximum velocity (V_{max}) of substrate activation by four orders of magnitude. Thrombin-activated factor VIIIa is composed of a heterotrimer of the 50-kDa A1-derived fragment, 43-kDa A2-derived fragment and the 73-kDa A3–C1–C2-derived fragment [13,14]. Thrombin activation of factor V releases the B domain to yield the active heterodimeric species, factor Va, composed of the amino-terminal derived 94-kDa heavy chain in a metal-ion association with the carboxy-terminal derived 74-kDa light chain [15–17].

Expression of factors VIII and V

Factor V is present both in plasma (8 mg/ml) and in the α granules of platelets [18]. It is primarily synthesized in megakaryocytes and hepatocytes and circulates in plasma as a 330-kDa single-chain polypeptide [19,20]. In contrast, the main physiological source of factor VIII is most likely the hepatocyte [21–24], and factor VIII is processed upon secretion from the cell to a heterodimer consisting of a carboxy-terminal derived light chain of 80 kDa in a metal-ion dependent association with a 200-kDa amino-terminal derived heavy chain fragment (Fig. 1). Factor VIII circulates in plasma (0.2 μg/ml) where the light chain is bound through noncovalent interactions to a primary binding site in the amino terminal of von Willebrand factor [25–28]. Von Willebrand factor interaction is required to increase the half-life of factor VIII in plasma from 2 to 12 h [29,30]. In addition, factors VIII and V both exhibit similar and extensive post-translational modifications, which include signal peptide cleavage, formation and conserved pairing of disulfide bonds [31–33], addition and complex modification of multiple asparagine-linked oligosaccharide residues [34,35], addition of multiple serine/threonine linked oligosaccharides within the B domain and sulfation of multiple tyrosine residues [36–39].

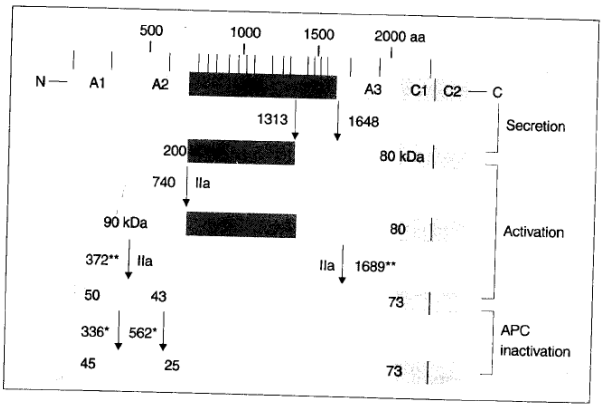

Fig. 1. Domain structure and processing of factor VIII. The structural domains of factor VIII are shown. Vertical bars represent potential amino-terminal linked glycosylation sites. Within the secretory pathway of the cell, factor VIII is cleaved within the B domain to generate a 200-kDa peptide and the 80-kDa light chain. The thrombin (IIa) and activated protein C (APC) cleavages that occur upon activation and subsequent inactivation are shown. **, necessary cleavages for activation; *, necessary cleavage for inactivation; aa, amino acid.

To date, there are no known primary or natural established cell lines that convincingly express factor VIII; thus, our knowledge of factor VIII expression is derived from interpretation of results from expression of the factor VIII complementary (c)DNA using expression vectors in transfected or virally infected mammalian cells. Factor VIII expression in these systems is two to three orders of magnitude lower than that observed with other genes, including factor V, using similar expression strategies. The biosynthesis and secretion of factor VIII were studied to elucidate the mechanism(s) responsible for the low level of factor VIII expression in transfected mammalian cells. At least three mechanisms were identified that limit factor VIII expression [40]: (1) factor VIII messenger (m)RNA is inefficiently expressed [41–44], (2) the primary translation product is inefficiently transported from the endoplasmic reticulum to the Golgi apparatus [45] and (3) high levels of von Willebrand factor are required in the conditioned medium to promote stable accumulation of factor VIII [34,41].

Initial studies demonstrated that expression of factor VIII from either transfected Chinese hamster ovary (CHO) [34] or baby hamster kidney (our unpublished results) cells required the presence of von Willebrand factor in the conditioned medium to stabilize the molecule upon secretion from the cell. In the absence of serum, a source for bovine von Willebrand factor, factor VIII was secreted as dissociated chains that were subsequently degraded in the conditioned medium. *In vitro* studies also demonstrated that von Willebrand factor promoted reconstitution of factor VIII activity from isolated heavy and light chains [46]. It appears that von Willebrand factor assists factor VIII secretion by two mechanisms: (1) to displace the light chain from the cell surface and (2) to promote heavy and light chain association. These results suggest that von Willebrand factor may interact with factor VIII to promote assembly of the heavy and light chains. The requirement for von Willebrand factor in the conditioned medium could be supplied by co-expression of von Willebrand factor within the same cell that expresses factor VIII. The co-expression of factor VIII and von Willebrand factor provided improved recovery of factor VIII in the conditioned medium, and this is now used as one method for the production of recombinant factor VIII for therapeutic use. In factor VIII and von Willebrand factor co-expressing cells, the genes for the two factors were equally well transcribed and their mRNAs were equally well translated. However, compared to von Willebrand factor, the steady-state level of factor VIII mRNA was reduced 60-fold and secretion efficiency of the factor VIII primary translation product was reduced eightfold [41]. Our recent studies have focused on the unique requirements that factor VIII displays for efficient secretion. These studies have identified a rate-limiting step at the stage of transport from the endoplasmic reticulum to the Golgi compartment.

Interaction of factor VIII with immunoglobulin-binding protein

Inefficient secretion of factor VIII correlates with interaction with the protein chaperone identified as the immunoglobulin-binding protein (BiP), which is the same as the glucose-regulated protein of 78 kDa (GRP78) [47] within the lumen of the endoplasmic reticulum [45,48]. This protein chaperone is expressed in all cell types and is a member of the heat-shock protein family that exhibits peptide-dependent ATPase activity [49]. BiP expression is induced at the transcriptional level by the presence of unfolded protein or unassembled protein subunits within the endoplasmic reticulum [50,51]. To elucidate the role of BiP in factor VIII secretion, we studied the effect of increasing and reducing cellular levels of the protein on factor VIII secretion. Overexpression of BiP inhibited factor VIII secretion [48] whereas the expression of antisense RNA to reduce BiP levels improved factor VIII secretion [52,53]. The specific activity of factor VIII secreted from cells with reduced BiP levels was not detectably altered, indicating that factor VIII can fold properly in cells with reduced BiP and that BiP interaction is not required for functional factor VIII secretion.

Electron microscopic examination of cells induced to express factor VIII at high levels demonstrated a dilation of the lumen of the endoplasmic reticulum as well as a large number of mitochondria juxtaposed to the rough endoplasmic reticulum membrane, which was not observed in control cells [54]. The appearance of the mitochondria at the site of factor VIII protein synthesis suggested that ATP may be uniquely required for factor VIII synthesis and secretion, possibly reflecting an ATP requirement for ATP-dependent release from BiP [49]. The requirement for ATP in factor VIII secretion was demonstrated by depleting intracellular ATP by treatment of cells with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) [55]. In a concentration-dependent manner CCCP uncouples oxidative phosphorylation [56]. Low concentrations of CCCP (10 µmol/l in [36]) completely inhibited factor VIII dissociation from BiP and inhibited secretion. In contrast, secretion of von Willebrand factor synthesized in the same cell, or factor V in an independent cell line, required 100–200 µmol/l CCCP to inhibit secretion. Thus, the block to factor VIII secretion at low concentrations of CCCP was not caused by a general defect in secretion. The ATP requirement for factor VIII se-

cretion was also demonstrated by expressing a BiP deletion mutant (amino acid residues 175–201) in CHO cells. This mutant BiP can bind peptides and ATP, but is defective in ATP hydrolysis [57]. Expression of this mutant protein completely inhibited factor VIII secretion, whereas secretion of macrophage colony-stimulating factor, a protein that does not bind BiP, was unaltered [58]. These results indicate that factor VIII secretion requires both a high intracellular ATP level and the ATPase activity of BiP.

Analysis of factor V expression indicated that secretion of this factor was 5- to 10-fold greater than that of factor VIII, that factor V did not detectably bind BiP and that factor V secretion was not inhibited by low concentrations of CCCP [36]. The availability of the homologous proteins factors V and VIII, which display significantly different secretion efficiencies, BiP interactions and ATP requirements for protein assembly and secretion, provides a unique approach to the dissection of factor VIII requirements for protein folding, chaperone interaction and efficient secretion.

Analysis of factor VIII deletion and factor VIII/factor V chimeric proteins demonstrated that sequences within the A1 domain of factor VIII inhibit secretion. Secretion of a chimeric factor VIII protein that had factor VIII residues 226–336 replaced by the homologous residues from factor V (residues 198–313) was increased 5- to 10-fold (Fig. 3 in [59]). However, the secreted chimeric protein did not display procoagulant activity, although it was susceptible to thrombin cleavage. The heavy and light chains of the 226–336 replacement were not associated and this could explain the absence of procoagulant activity. These results indicate that between residues 226 and 336 within factor VIII, there are sequences that either actively retain factor VIII in the endoplasmic reticulum or otherwise affect protein folding to prevent factor VIII secretion, and are consistent with recently reported results from Ortel *et al.* [60]. In addition, sequences within this 226–336 region are required for factor VIII heavy and light chain association.

Comparison of the 226–336 region that inhibits factor VIII secretion to the homologous residues in factor V provided insight into amino acids potentially involved in BiP binding and factor VIII protein folding [59]. Aliphatic amino acids (such as Leu) and aromatic amino acids (such as Phe) are enriched in peptides that have highest affinity for binding to BiP [61,62]. Residues Leu303 to Phe309 contain a hydrophobic cluster where seven out of 11 amino acid residues are Leu or Phe. Site-directed mutagenesis was performed to mutate all seven Leu and Phe residues in the potential BiP binding pocket to Ala; however, the resultant molecule was not secreted. Subsequently, individual Phe residues were mutated to the respective amino acid residues in factor V. The activity of the Phe309Ser mutant was reproducibly increased over twofold in several transfection experiments [63]. The increased activity of the Phe309Ser mutant correlated with a twofold increase in factor VIII antigen, indicating a specific activity similar to wild-type factor VIII. Metabolic labeling with [^{35}S]-methionine indicated that the increased activity of the Phe309Ser mutant correlated with increased secretion compared to wild-type factor VIII (Fig. 2a). Mutations at other adjacent residues (Phe293Ser, Phe306Try) did not improve secretion. The increased secretion of the Phe309Ser mutant factor VIII was almost equal to that of the 226–336 factor VIII/factor V hybrid molecule, indicating that this single amino acid was primarily responsible for the reduced secretion efficiency of factor VIII compared to factor V.

Stably transfected CHO cell lines were derived that express either the 226–336 factor VIII/factor V chimeric molecule or the Phe309Ser mutant factor VIII in order to study its BiP interaction. Of 35 original transfected CHO cell clones selected for dihydrofolate reductase expression, the selectable marker used in the transfection, five clones were obtained that express significant levels of Phe309Ser mutant factor VIII (greater than 1 U/ml per 10^6 cells per day). Two of these clones express greater levels of factor VIII compared to the original 10A1 cell line that was obtained by screening over 1000 original transfected cell clones [34]. Thus, at this initial stage of selection in low concentrations of methotrexate, the mutation permits high-level factor VIII expression to be obtained more readily. However, further selection in higher concentrations of methotrexate did not yield significantly greater levels of factor VIII expression. However, previous experience had indicated that factor VIII expression at greater than 1 U/ml becomes limited due to insufficient amounts of von Willebrand factor in the serum-containing medium [34]. Thus, it may be necessary to co-express von Willebrand factor in order to obtain further increases in the level of Phe309Ser mutant factor VIII secretion. Although the Phe309Ser mutant factor VIII was more easily expressed and secreted, co-immunoprecipitation experiments demonstrated that the Phe309Ser mutant factor VIII and the 226–336 hybrid both displayed interaction with BiP.

For a more careful study of the requirement for BiP interaction in the secretion of Phe309Ser mutant factor VIII and the 226–336 factor VIII/factor V hybrid, the effect of ATP depletion was studied by treatment with CCCP [63]. This analysis demonstrated that the 226–336 hybrid molecule required high levels of CCCP to inhibit its transport, similar to factor V, whereas transport of wild-type factor VIII was inhibited by low con-

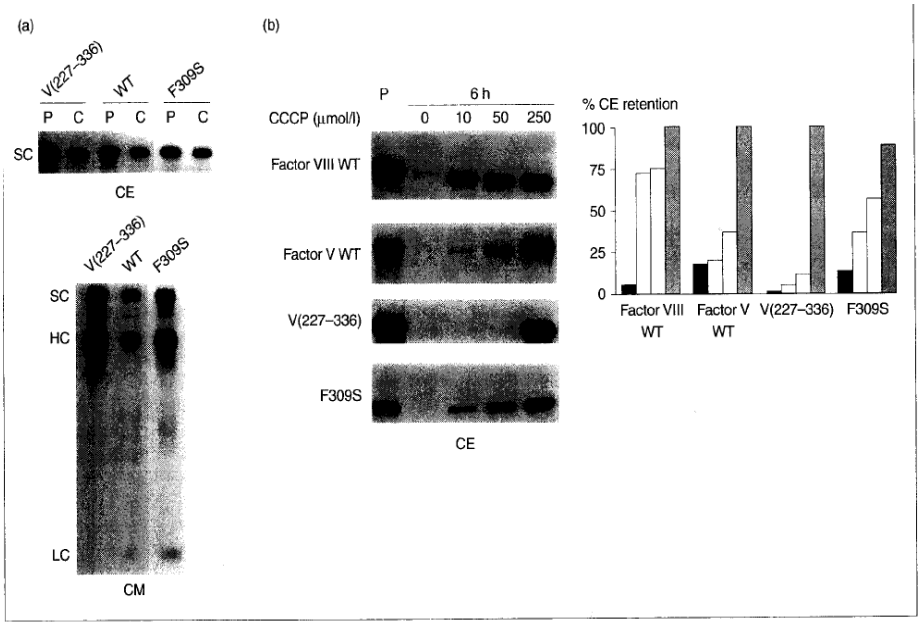

Fig. 2. Mutation of Phe309 increases secretion efficiency and reduces the ATP requirement. (a) Expression vectors were transfected into COS-1 cells. After 60 h the cells were pulse-labeled with [^{35}S]-methionine for 15 min and chased in complete medium for 4 h. Cell extracts (CE) and conditioned medium (CM) were immunoprecipitated with the antifactor VIII heavy-chain monoclonal antibody. The migration of the single chain (SC), heavy chain (HC) and light chain (LC) are shown. P and C represent pulse and chase for cell extracts. WT, wild type. (b) Chinese hamster ovary cells expressing wild-type (WT) factor VIII, factor V, the V(227–336) hybrid and the F309S mutant factor VIII were pulse-labeled with [^{35}S]-methionine for 15 min and chase was performed for 6 h in complete media containing increasing concentrations of carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Cell extracts were prepared for immunoprecipitation and analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography (left). Band intensities were quantified and presented relative to the proportion of pulse-labeled (P) protein for each cell line (right). The concentrations of CCCP are displayed by bars: 0 (black), 10 μmol/l (white), 50 μmol/l (light grey) and 250 μmol/l (dark grey).

centrations of CCCP (Fig. 2b). In addition, the Phe309Ser mutant factor VIII displayed inhibition at intermediate levels of ATP depletion. These results indicate that the Phe309Ser factor VIII molecule binds BiP, but with an apparent lesser affinity, and that this interaction does not require high levels of ATP for release. These results show that a single amino acid change within factor VIII results in a molecule that is more efficiently secreted, possibly due to a lesser interaction with BiP, and retains full functional activity. This mutant may prove useful in gene therapy strategies where inefficient secretion of factor VIII and its intracellular accumulation within the secretory pathway may be detrimental to cell survival.

The BiP-binding motif is best characterized by bulky aromatic or hydrophobic residues and is consistent with nuclear magnetic resonance data showing that peptides bind to DnaK, the BiP homologue in *Escherichia coli*, in an extended conformation [64]. The recent structural determination of the peptide-binding region of DnaK bound to a peptide [65] demonstrates that a single hydrophobic residue in the middle of the bound peptide exhibits most contacts with a deep groove in the peptide-binding site of DnaK. We speculate that Phe309 is bound deep within the peptide-binding cleft. Mutation of this Phe to Ser would significantly reduce, but may not eliminate, the hydrophobic interactions with BiP.

The role of copper ion in factor VIII folding and chain assembly

Each of the A domains in factor VIII have conserved disulfide bridges and can be subdivided into two subdomains (subdomains d1 through d6 for the three

A domains in factor VIII, factor V, and ceruloplasmin). Each d subdomain resembles the β-barrel folded structure of the ancient copper ion binding protein cupredoxin [66]. The blue copper-containing proteins and proteins related to them form a heterogeneous group that extend from the small blue proteins in bacteria and plants to the blue oxidases in plants and mitochondria, and to the coagulation factors V and VIII in vertebrate plasma [67]. These proteins contain copper ions of three types that are defined by the amino acid ligands that constitute the binding site. The blue color characteristic of the blue copper-containing proteins is attributed to the presence of a type-1 copper binding site with characteristic spectral and redox properties [68]. The blue oxidases and related proteins, which include the plant proteins laccase and ascorbate oxidase, and the plasma protein ceruloplasmin, contain an intensely blue type-1 Cu(II) ion with absorption in the visible region, a trinuclear cluster consisting of a pair of magnetically coupled type-3 copper ions characterized by a strong absorption in the near-ultraviolet region and the absence of an electron paramagnetic resonance (EPR) signal, and a magnetically isolated type-2 Cu(II) ion having an undetectable absorption. The type-1 and type-2 Cu(II) ions have unique EPR spectra that disappear and absorption properties that change upon reduction of the copper ion to Cu(I) [69]. Laccase and ascorbate oxidase perform oxidation reactions by transferring four electrons from a reducing substrate to a molecule of oxygen that is subsequently reduced to water. Sequence alignments and three-dimensional structures support the hypotheses that the small blue proteins and the blue oxidases are homologous [70] and evolved from a common origin through gene duplication and divergence [67,71]. The evolutionary and structural conservation of the A domains between the coagulation factors and the blue oxidases suggests a role for copper ion binding in the coagulation factors. Indeed, approximately 1 mol copper ion/mol protein was directly detected in factor VIII by atomic absorption spectroscopy [7] and in factor V by atomic absorption and atomic emission spectroscopy [6]. The presence of copper ion in factor VIII was specifically associated with the functionally active heterodimeric factor VIII species [7].

We have studied the copper ion oxidation state and the ligands that coordinate copper ion in factor VIII by atomic absorption spectroscopy, EPR and site-directed mutagenesis and expression of the factor VIII cDNA [72]. Whereas copper ion was not detected in intact recombinant factor VIII, ethylenediaminetetraacetic acid (EDTA) dissociation of the chains yielded an EPR signal consistent with 1 mol Cu(II) per mol of active protein. These results support the theory that a single molecule of reduced copper [Cu(I), which is EPR-silent] is buried within intact factor VIII and is released and oxidized to Cu(II) upon treatment with EDTA. Cu(I), and not Cu(II), was able to reconstitute factor VIII activity from dissociated heavy and light chain subunits, demonstrating a requirement for Cu(I) in factor VIII heavy- and light-chain assembly and function.

Two potential consensus type-1 copper ion binding sites occur within factor VIII, composed of the ligands His265-//-Cys310—His315—Met320 in the A1 domain and ligands His1954-//-Cys2000—His2005—Met2010 in the A3 domain. In addition, Pemberton *et al.* [73] recently proposed a model based on the structural determination of ceruloplasmin for the structure of the three A domains of factor VIII. In this model, a type-2 copper ion binding site composed of residues His99 and His1957 is proposed to support the A1 and A3 domain metal-ion association. The importance of these potential copper ion binding sites was tested by studying the effect of site-directed mutation of the specific amino acid ligands [72]. Factor VIII having the conservative mutation of Cys310Ser (within the type-1 copper binding site in the A1 domain) was inactive, partially defective for secretion from the cell, and the heavy and light chains of the secreted protein were not associated. In contrast, factor VIII mutants Cys2000Ser (within the type-1 site in the A3 domain) and His1957Ala (within the type-2 site in the A3 domain) displayed secretion, assembly and activity similar to wild-type factor VIII. Finally, mutant His99Ala (within the type-2 site in the A1 domain) was partly defective for secretion; however, the resultant secreted protein had detectable heavy and light chain association and activity. Taken together, these results are consistent with the hypothesis that the type-1 copper ion binding site within the A1 domain is essential for efficient secretion, heavy and light chain association, and functional activity and suggest that a Cu(I) ion is occupying this site.

It is interesting that the potential high-affinity BiP binding site (residue Phe309) is adjacent to a ligand within the type-1 copper binding site (residue Cys310) and may suggest an association between copper interaction and factor VIII protein folding. The recent structural model of the factor VIII A domains proposed by Pemberton *et al.* [73] suggests that the the type-1 copper binding site in the A1-domain is adjacent to the A2-domain. Since the proposed location of the phenyl side chain of Phe309 is 3.7 Å from Phe536 in the A2 domain, there may be a potential hydrophobic interaction between the A1 and the A2 domains. However, mutation of Phe309Ser did not affect the A1 and A2 domain interaction, whereas this mutation did weaken the metal-ion association between the A1 domain and the A3 domain, as indicated by increased sensitivity to

EDTA-induced metal-ion chelation [63]. Therefore, the mutagenesis data do not support location of the type-1 copper ion binding site within the A1 domain as proposed by Pemberton *et al.* [73]. Further experimental evidence is required to elucidate the validity of the structural model.

Factor VIII interaction with lectin-binding protein chaperones calnexin and calreticulin

Calnexin (also known as p88 or IP90) and calreticulin are two recently characterized molecular chaperones localized to the lumen of the endoplasmic reticulum which associate transiently with many soluble and membrane glycoproteins during folding and subunit assembly [74,75]. The most recent data indicate that calnexin and calreticulin promote folding of associated glycoproteins [76]. Prolonged association with calnexin/calreticulin is observed when proteins are unfolded, misfolded or unable to oligomerize. Studies to date demonstrate that the calnexin/calreticulin recognition motif within a newly synthesized protein is at least partly determined by the structures of asparagine-linked oligosaccharides. Upon translocation into the lumen of the endoplasmic reticulum, a core unit of 14 saccharides (Glc3Man9GlcNAc2) is added to selective asparagine residues. Immediately after, sequential trimming of the three terminal glucose residues occurs by the action of glucosidases I and II. Binding to calnexin/calreticulin requires monoglucosylated asparagine-linked oligosaccharides, an intermediate in the trimming process [77–79]. Studies using glucosidase inhibitors and glucosidase-deficient cell lines confirmed the requirement for glucose trimming for the calnexin/calreticulin interaction [80,81]. However, some polypeptides that contain no amino-terminal linked oligosaccharides associate with calnexin [82–84], indicating that calnexin binding may occur in more than one way. How do calnexin and calreticulin selectively bind unfolded glycoproteins? A UDP-glucose:glycoprotein glucosyltransferase (GT) can re-glucosylate de-glucosylated high-mannose containing oligosaccharides [85,86]. Interes- tingly, this glucosyltransferase selectively uses unfolded glycoproteins, as opposed to native glycoproteins, as substrates [87,88]. Therefore, it was proposed that the specificity for the calnexin and calreticulin interaction for unfolded glycoproteins and subsequent retention within the endoplasmic reticulum is imparted by the ability of this glycosyltransferase to discriminate between unfolded and folded glycoproteins [89]. Under this hypothesis, only unfolded glycoproteins would be glucosylated and subsequently interact with calnexin and calreticulin and be retained within the endoplasmic reticulum compartment.

Do calnexin and calreticulin influence the secretion of factor VIII and/or factor V? The interaction of calnexin and calreticulin with factor V and factor VIII transiting the secretory pathway was studied using specific immunoprecipitation with antifactor VIII, antifactor V, anticalnexin and anticalreticulin antibodies to detect association by co-imunoprecipitation. Immunoprecipitation of factor VIII-expressing CHO cells with antifactor VIII antibody detected the primary translation product of 300 kDa (Fig. 3a; lane 2). Immunoprecipitation of cell extract with anticalnexin antibody precipitated calnexin, but also co-immunoprecipitated a significant portion of the factor VIII primary translation product (Fig. 3a; lane 6). Incubation in the presence of castanospermine, a glucosidase I and II inhibitor, reduced the amount of factor VIII interaction with calnexin (Fig. 3a; lane 7). These results suggest that factor VIII can interact with calnexin in a manner that requires glucose trimming of the oligosaccharide core structure. Similar analysis of factor V-expressing CHO cells demonstrated factor V synthesis as a polypeptide which migrated at 330 kDa upon immunoprecipitation with antifactor V antibody (Fig. 3a; lane 4). However, immunoprecipitation with anticalnexin antibody did not detect factor V in a complex with calnexin in the factor V-expressing CHO cells (Fig. 3a; lane 8).

Similar analysis with anticalreticulin antibody demonstrated that the majority of both wild-type factor VIII and wild-type factor V interact with calreticulin (Fig. 3b). These results indicate that these two homologous glycoproteins exhibit distinctly different interactions with calnexin, whereas they exhibit similar extensive interactions with calreticulin. Further pulse-chase experiments indicated that factor VIII initially interacts with BiP and calreticulin upon translocation into the lumen of the endoplasmic reticulum and subsequently interacts with calnexin, similar to results with vesicular stomatis virus G glycoprotein and thyroglobulin [90,91]. In addition, ternary complexes between factor VIII, BiP and calnexin were not detected, suggesting that intracellular factor VIII is bound either to BiP or to calnexin (data not shown).

Since calnexin is known to specifically bind monoglucosylated oligosaccharide structures on glycoproteins, and the B domain of factor VIII is the most extensively glycosylated region of the molecule, we conducted tests to determine whether the B domain was responsible for the calnexin interaction. We evaluated the calnexin interaction of a B-domain deletion molecule that is secreted as a functional factor VIII molecule [92]. Immunoprecipitation analysis of extracts from [^{35}S]-methionine-labeled cells demonstrated that factor VIIIΔB had significantly reduced binding to calnexin and calreticulin, as shown by comparison of the

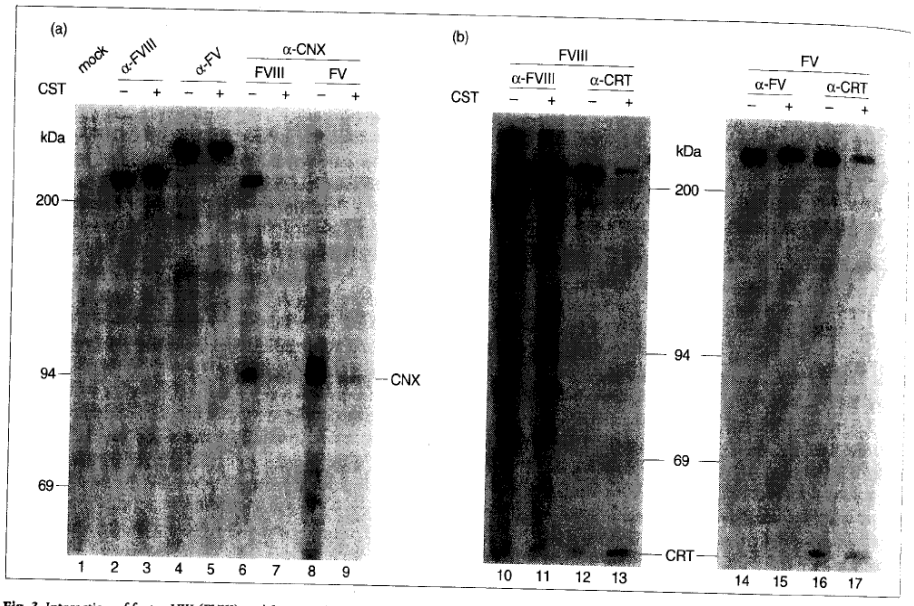

Fig. 3. Interaction of factor VIII (FVIII) and factor V (FV) with protein chaperones calnexin (CNX) and calreticulin (CRT). Calnexin and calreticulin interactions with factor VIII and factor V proteins were analyzed by labeling Chinese hamster ovary cells for 2 h with [^{35}S]-methionine. Mock represents cells that did not receive antibody. Molecular weight markers are shown at the side of the radiographs. Cell extracts were prepared, immunoprecipitated with (a) antifactor VIII, antifactor V and anticalnexin, or (b) anticalreticulin antibody, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. Where indicated, the cells were treated with the glucosidase I and II inhibitor castanospermine (CST).

antifactor VIII, anticalnexin and anticalreticulin immunoprecipitations (data not shown). These results suggest that one primary determinant of calnexin binding was contained within the factor VIII B domain and that this domain displayed significantly different calnexin-binding properties from those of the factor V B domain.

The fundamental question concerning the significance of the calnexin/calreticulin interaction is whether this facilitates or inhibits factor VIII secretion. This question was addressed by inhibition of glucose trimming, a process that inhibits calnexin and calreticulin interaction, by two methods. First, cells were treated with deoxynojirimycin, an inhibitor of glucosidases I and II. The factor VIII activity secreted was reduced to 10% in the presence of this inhibitor. In contrast, deoxynojirimycin had no effect on the amount of factor V secreted into the conditioned medium. The requirement for glucosidase I in factor VIII secretion was also studied by transient transfection of glucosidase I-deficient CHO cells (Lec23) that were previously isolated by lectin resistance by Pamely Stanley at the Albert Einstein University [93]. Whereas secreted factor VIII was significantly reduced in the Lec23 cells (to less than 10%) compared with the wild-type CHO cells, the secretion of factor VIIIΔB was partly reduced (approximately 50%) and the activity of factor V was only slightly reduced. Quantitation of factor VIII protein antigen by enzyme-linked immunosorbent assay (ELISA) indicated that the amount of factor VIII protein secreted was reduced with no change in the specific activity. These results show selective inhibition of factor VIII secretion compared to that of factor V in the absence of glucose trimming and suggest that calnexin interaction is required for secretion of wild-type factor VIII. The results also suggest that calnexin is also required, although to a lesser extent, for factor VIIIΔB secretion.

The fact that B-domain deleted factor VIII is expressed at a greater level than wild-type factor VIII in a number of different cell culture systems [45,92,94] is one reason why most gene therapy studies on hemophilia A have used the B-domain deletion molecule. B-domain deleted factor VIII is expressed at a greater level

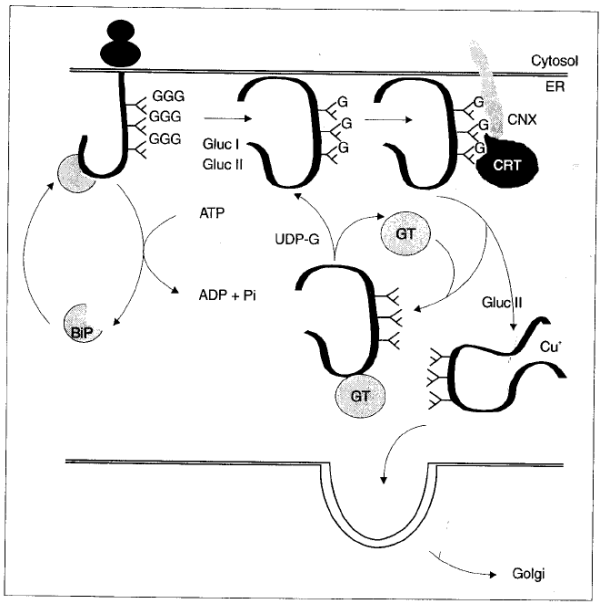

Fig. 4. Model of chaperone interactions in factor VIII folding, assembly and transport within the early secretory pathway. The sequential interaction of newly synthesized factor VIII with immunoglobulin-binding protein (BiP), calnexin (CNX) and calreticulin (CRT) is shown. After ATP-hydrolysis dependent release from BiP, factor VIII is subject to glucosidase (Gluc) I and II action which generates a monoglucosylated intermediate (G) that can then interact with calnexin and/or calreticulin. Unfolded factor VIII is proposed to be a substrate for the UDP-glucose: glycoprotein glucosyltransferase (GT) to form a monoglucosylated ligand to promote another round of calnexin and/or calreticulin binding. ER, endoplasmic reticulum; Pi, inorganic phosphate.

because of a large increase in its mRNA level and, consequently, in protein translation [95]. However, the secretion efficiency, that is, the amount of protein secreted from the cell compared to the total amount of protein synthesized, is actually less than that for wild-type factor VIII. In other words, there is a larger percentage of B-domain deleted factor VIII left in the cell after 4 h compared to wild-type factor VIII. These observations are consistent with the B domain having a positive influence on factor VIII transport out of the cell which is dependent on the calnexin interaction.

Conclusions

Our studies indicate that factor VIII secretion is dependent upon the productive interaction with the protein chaperones, BiP, calnexin and calreticulin. Our present results are summarized as a model in Fig. 4. Upon translocation of factor VIII into the lumen of the endoplasmic reticulum, factor VIII immediately interacts with BiP through a primary binding site within the A1 domain. In reactions that require ATP hydrolysis for release from BiP and glucosidases I and II for glucose trimming, factor VIII molecules containing monoglucosylated structures interact with calnexin and calreticulin. Previous studies suggest that polypeptide folding occurs while proteins are associated with calnexin [76]. Our results suggest that the calnexin/calreticulin interaction contributes to factor VIII secretion, possibly by promoting release of factor VIII from BiP and/or promoting association of the heavy and light chains of factor VIII. Coordinated release from BiP and interaction with calnexin and/or calreticulin may be required for efficient Cu(I)-dependent association of the A1 domain with the A3 domain. Glucosidase II would subsequently remove the last glucose and promote re-

lease of factor VIII from calnexin/calreticulin. However, if factor VIII is improperly folded it will activate the UDP-glucose:glycoprotein glucosyltransferase to reglucosylate oligosaccharide cores within the B domain and subsequently promote another round of calnexin/calreticulin interaction. In this manner, only fully folded factor VIII may escape the chaperone-mediated retention mechanisms and finally be transported to the Golgi compartment and out of the cell. In contrast to factor VIII, factor V has evolved a pathway of protein folding that is very efficient and does not rely on BiP to prevent the secretion of unfolded intermediates or calnexin to assist its secretion. Although factor V and factor VIII display very similar tertiary structures, post-translational modifications including amino-terminal linked glycosylation, and proteolytic cleavages required for activation and inactivation, they display significantly different requirements for secretion from the cell. To date, there have been a number of reports of model proteins that exhibit interactions with calnexin and/or calreticulin; however, the unique differences between the homologous proteins factor V and factor VIII in their interaction with protein chaperones provide a unique and powerful model system to elucidate the functional roles of calreticulin and calnexin.

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Minireview

The Making of a Professional Secretory Cell: Architectural and Functional Changes in the ER during B Lymphocyte Plasma Cell Differentiation

Luigina Tagliavacca[1,2], Tiziana Anelli[1,2], Claudio Fagioli[2], Alexandre Mezghrani[2,3], Elena Ruffato[2] and Roberto Sitia[1,2,*]

[1] Università Vita-Salute, San Raffaele, Italy
[2] DIBIT-HSR Scientific Institute, I-20132 Milano, Italy
[3] IGH, CNRS UPR 1142, F-34396 Montpellier, France

*Corresponding author

B lymphocytes are small cells that express antigen receptors and secrete little if any IgM. Upon encounter with antigen, they differentiate into short-lived plasma cells, which secrete large amounts of polymeric IgM. Plasma cell differentiation entails a massive development of the endoplasmic reticulum to sustain high levels of Ig production. Recent findings suggest a role for the unfolded protein response in orchestrating the architectural and functional changes during terminal plasma cell differentiation.

Key words: B lymphocyte differentiation / Endoplasmic reticulum / ER-associated degradation / IgM secretion / Plasma cells / Unfolded protein response.

Introduction

Textbooks often utilize antibody-secreting plasma cells as prototypes of professional secretory cells. Plasma cells are the end-stage effectors of the humoral immune response, in charge of producing immunoglobulins (Ig). They fulfill this task with extraordinary efficiency, each of them being capable to release thousands Ig molecules per second. To achieve these standards, they expand the organelles devoted to protein synthesis, transport and secretion, and in particular the endoplasmic reticulum (ER) where Igs are synthesized, folded and assembled. Although several subtypes of plasma cells exist, they share a number of common features, such as the presence of a highly developed ER and a rather short life span. Whilst the former characteristic is essential to mount an immune response, the latter is important to limit it.

B Cell Development and the Unfolded Protein Response

Plasma cells originate from B lymphocytes. The latter are long-lived cells, with a very small cytoplasm and scarce ER cisternae generally limited to the perinuclear area. B cells express surface Ig as antigen receptors (B cell receptors; BCRs), but secrete little if any antibodies. Secreted Ig could compete with the BCR at low antigen concentration, thus hampering the primary immune response. Upon encounter with the cognate antigen, a complex proliferative and differentiative process is activated, leading to expansion of the antigen-specific clones, generation of memory cells and affinity maturation (Lanzavecchia and Sallusto, 2002; Calame *et al.*, 2003). To provide an effective defense against the antigen, some cells promptly differentiate into plasma cells, entirely reshaping their architecture in anticipation of their novel role as Ig-secretors.

How is this spectacular metamorphosis coordinated? What controls the biogenesis of the ER? Several lines of evidence point to a role for the Unfolded Protein Response (UPR) pathway in the regulation of terminal B cell differentiation. In all eukaryotic cells, ER chaperones are transcriptionally up-regulated by conditions that affect protein folding in the ER (*e.g.* pharmachological blockade of glycosylation or disulfide bond formation, synthesis of mutated or orphan proteins) and lead to the accumulation of aberrant proteins. In mammalian cells, the UPR is started by three ER-localized trans-membrane proteins ATF6, Ire1 and PERK (Figure 1), that act as sensors of unfolded proteins, monitoring the levels of free binding protein (BiP), one of the most abundant and conserved ER chaperones (Bertolotti *et al.*, 2000; Mori, 2000; Kaufman, 2002). Activated ATF6 and Ire1 drive the sequential expression of many genes involved in ER protein folding and ER associated degradation (ERAD), respectively. PERK provides another form of relief on ER stressed cells: by phosphorylating eIF2α it attenuates protein synthesis, thus reducing the load on the protein folding machineries. Translational competence is rescued by GADD34, an eIF2α-specific phosphatase probably induced by PERK itself (Novoa *et al.*, 2001, 2003). This pathway also activates ATF4, causing increased expression of genes involved in aminoacid import and redox regulation (Harding *et al.*, 2003), and the CHOP/GADD153 transcription factor involved in apoptosis (Figure 1). The capability of inducing

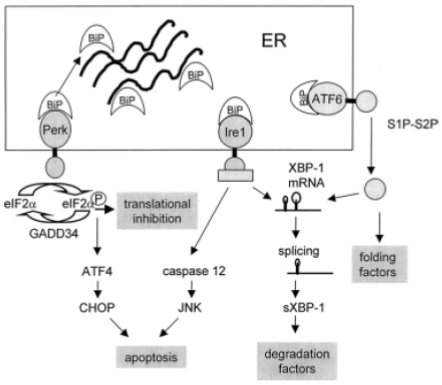

Fig. 1 The Unfolded Protein Response.
When misfolded proteins accumulate in the ER, three ER localised membrane proteins are activated, Perk, Ire1 and ATF6. Activation seems to be mediated by the release of BiP from the ER transmembrane proteins (see Harding *et al.*, 2002 and Kaufman, 2002, for reviews).
Upon activation, Perk phosphorylates eIF2α causing translation inhibition. Subsequently, ATF4 is up-regulated increasing the transcription of genes involved in amino acid import, redox regulation and apoptosis (CHOP; see Harding *et al.*, 2003 and references therein). GADD34, also up-regulated by the Perk-dependent pathway, dephosphorylates eIF2α, thus rescuing translation competence (Novoa *et al.*, 2003). ATF6 activation requires cleavage by S1P and S2P, two Golgi-localised proteases (Ye *et al.*, 2000; Shen *et al.*, 2002). Cleaved ATF6 (p50) increases the transcription of ER chaperones and enzymes, mainly implicated in protein quality control and folding. It also induces XBP-1 transcription. Activated Ire1 cleaves XBP-1 mRNA. This non-classical splicing occurs during *in vitro* B cell differentiation. Spliced XBP-1 (XBP-1s) increases transcription of genes implicated in glycoprotein degradation (EDEM; Yoshida *et al.*, 2003).

cell death probably explains why UPR pathways are key suspects in the pathogenesis of many conformational diseases (Oyadomari *et al.*, 2002; Yoneda *et al.*, 2002).

Physiologically, the UPR is thought to monitor the demand placed on the ER protein folding machinery and allow cells to promptly adapt to novel developmental requests (Shen *et al.*, 2001; Yoshida *et al.*, 2001; Calfon *et al.*, 2002; Ma and Hendershot, 2003). Therefore, the remarkable increase in Ig production that accompanies B to plasma cell differentiation could drive the expansion of the ER *via* the UPR (Ma and Hendershot, 2003). A clear hint for such a role came from the observation that XBP-1$^{-/-}$ mice lack plasma cells and serum Igs, despite having normal numbers of B lymphocytes (Reimold *et al.*, 2001). It is thus clear that XBP-1 is essential for terminal B cell differentiation. XBP-1 is a ubiquitously expressed transcription factor whose activity is regulated at least at two levels. Its transcription is stimulated by ATF6 and by IL-4. However, XBP-1 mRNA encodes a protein that is essentially inactive. Removal of a 26 base intron by a non-spliceosomal pathway causes a frame shift producing an active XBP-1 in which a DNA activation domain is placed downstream the DNA binding cassette. Intron removal in XBP-1 transcripts is mediated by activated Ire1 (Shen *et al.*, 2001; Yoshida *et al.*, 2001; Calfon *et al.*, 2002). The products of the 'spliced' XBP-1 mRNAs (XBP-1s) drive the transcription of EDEM and other genes possibly involved in ERAD (Yoshida *et al.*, 2003). It is not yet clear what is the role, if any, of unspliced XBP-1 protein. Since XBP-1 lacks the DNA activation domain, this highly unstable protein may act as an inhibitor. The two-step regulation of XBP-1s results in its delayed appearance with respect to ATF6. This circuit times events during the UPR (Yoshida *et al.*, 2003), so that factors involved in folding (controlled by ATF6) precede those involved in degradation (dependent upon XBP-1s). It therefore appears that cells try first to fix the problem, and then activate protein degradation. If also this combined response fails, apoptosis is activated so as to limit the damages that abnormal proteins could cause to the organism. Since mice lacking ATF6 are not available, it has not been possible yet to establish the role of this arm of the UPR in B cell differentiation.

Recent findings exploiting a B-cell lymphoma that can be induced to differentiate *in vitro* indicate that the expansion of the ER begins before the increase in Ig synthesis. Many mitochondrial proteins and enzymes involved in amino acid and membrane synthesis are also increased in this initial phase. The synthesis of Ig and J chains sharply increases only after 2 to 3 days of stimulation, concomitantly with the appearance of XBP-1s (van Anken *et al.*, 2003). It follows that, at least in the first days of differentiation, the synthesis of ER proteins is stimulated by mechanisms that are independent from the production of the main cargo molecules. It is still unclear what regulates the increased production of BiP, calreticulin and other ER resident proteins in the first days of differentiation in response to lipopolysaccharide. In contrast, the appearance of XBP-1s correlates well with the onset of Ig production, suggesting cargo dependent feed-forward mechanisms based on UPR circuits. Recent results obtained with transgenic mice in which the expression of μ chains can be ablated upon Cre activation confirm that IgM production controls the accumulation of active XBP-1s (Iwakoshi *et al.*, 2003). Also in LPS activated splenocytes, XBP-1s becomes detectable after three days (Calfon *et al.*, 2002). Taken together, these results suggest that XBP-1s comes into action in the terminal stages of plasma cell differentiation. It remains to be seen whether unspliced XBP-1 transcripts, that are induced by IL-4 or following LPS activation of splenocytes (Harding *et al.*, 2002), play a role in the early phases of B cell activation. It will be of interest to better characterize morphologically and functionally the stage at which B cells accumulate in XBP-1$^{-/-}$ mice.

The amino terminal domains of Ire1 and PERK show extensive similarities: they both bind BiP and are func-

tionally interchangeable (Liu *et al.*, 2000). This creates a paradoxical situation for plasma blasts and plasma cells. On one hand, these cells require XBP-1s, and hence an active Ire1 pathway. On the other, PERK-mediated translational attenuation would be detrimental for their mission, that is massive antibody production. Mechanisms could exist that activate preferentially Ire1, possibly involving the hsp40-like co-chaperones that reside in the ER. Otherwise, the activation of GADD34 pathway could allow protein synthesis to proceed while Ire1-dependent XBP-1 production takes place.

Since XBP-1s drives the expression of ERAD components, such as EDEM (Yoshida *et al.*, 2003) and ER mannosidase I (Molinari *et al.*, personal communication), it seems that plasma blasts and plasma cells place a high demand on efficient degradative pathways. In IgM producing cells, this may reflect the difficulties inherent to the processes of assembling and polymerizing this isotype. Indeed, a fraction of μs chains are degraded by proteasomes even in IgM secreting myeloma transfectants (Fra *et al.*, 1993; Fagioli and Sitia, 2001).

ER Redox Control and Thiol-Mediated Retention

Many ER synthesized proteins contain disulfide bonds that are essential for their structure and function. Formation of disulfide bonds require that oxidative conditions be generated and maintained in the ER despite the continuous entry of newly synthesized polypeptides with reduced cysteines. In eukaryotic cells, members of the Ero1 family play an essential role in oxidative protein folding, transferring oxidative equivalents to cargo proteins primarily *via* PDI (Frand and Kaiser, 1998; Frand and Kaiser, 1999; Mezghrani *et al.*, 2001). Whilst a single ERO1 gene exist in yeast, two isoforms (Ero1α and β) are produced by mammalian cells. The former is produced by most cell types, while the expression of Ero1β is limited to certain tissues and is induced during the UPR (Cabibbo *et al.*, 2000; Pagani *et al.*, 2000). If the oxidative power is insufficient to cope with all newly made proteins, as might be the case of professional secretors such as pancreas or plasma cells, reduced cargo might accumulate and induce the synthesis of Ero1β *via* UPR pathways. However, disulfide bonds are not only formed in the ER lumen, but also extensively isomerised in folding intermediates and reduced in ERAD substrates before dislocation to the cytosol (Tortorella *et al.*, 1998; Fagioli *et al.*, 2001; Fassio and Sitia, 2002). Since these reactions are thought to be catalyzed by reduced PDI (Tsai *et al.*, 2002), Ero1 activity must be tightly controlled. The mechanisms that regulate the levels and function of Ero1α and prevent cells from excessive oxidation remain to be understood. Another open question is what is the role of the numerous oxidoreductases that populate the ER, and whether their redox state is also controlled, directly or indirectly, by Ero1 proteins.

Developmental Control of IgM Secretion

In compliance to the rules of ER quality control (Ellgaard and Helenius, 2003), transport to the Golgi and secretion are restricted to IgM polymers (Davis *et al.*, 1989; Sitia *et al.*, 1990; Brewer *et al.*, 1994). Folding and assembly intermediates are retained in the ER, and eventually degraded by cytosolic proteasomes. IgM polymerization proceeds stepwise. First, Ig-μ chains are assembled with L chains. In all Ig isotypes, the first constant domain of H chains binds BiP, thus preventing transport to the Golgi of unassembled chains. The CH2 domain is responsible for the formation of H2L2 complexes (see Hendershot and Sitia, 2003, for review). Whilst this is sufficient to negotiate transport of IgG or IgE (called in the immunological jargon 'monomeric' Ig), IgM and IgA undergo a further level of quality control that ensures further assembly into covalent polymers. The penultimate cysteine in the tailpieces of secretory μ and α chains acts as a three-way switch, mediating assembly, retention and degradation of unpolymerized $\mu s_2 L_2$ or $\alpha s_2 L_2$ subunits (Fra *et al.*, 1993; Guenzi *et al.*, 1994). This thiol-based quality control mechanism is exploited to regulate the expression of many secretory, transmembrane and GPI-anchored proteins (Kerem, *et al.*, 1993; Capellari, *et al.*, 1999). Its stringency can be modulated by the amino acid context surrounding the unpaired cysteine(s) involved: for instance, the presence of vicinal acidic residues weakens retention. This allows some unassembled light chains and monomeric IgA to be secreted by plasma cells (Guenzi, *et al.*, 1994; Reddy *et al.*, 1996).

Noteworthy, this basic mechanism is employed during B cell development to dictate the fate of secretory IgM. Unlike plasma cells, in fact, B cells are unable to form polymers; as a result, they degrade virtually all secretory μ and α chains (Figure 2). It has long been thought that the failure of B cells to polymerize and secrete IgM or IgA was due to the absence of J chains. However, the ectopic expression of J chains in B cells is not sufficient to induce efficient polymerization (Randall *et al.*, 1992; C.F. and

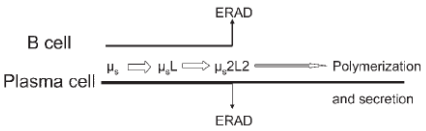

Fig. 2 Differential Fate of Secretory IgM in B and Plasma Cells. While both B and plasma cells can synthesize and assemble secretory μ chains (μ_s) into μ_sL and μ_s2L2 subunits, further polymerization into secretion competent polymers is slower and restricted to plasma cells. As a result, B cells retain and degrade virtually all secretory μ chains by thiol-dependent mechanisms (Sitia *et al.*, 1990; Fra *et al.*, 1993). Reduction of the inter-chain disulfide bonds linking μ and H chains precedes dislocation and degradation by cytosolic proteasomes (Fagioli *et al.*, 2001). These observations imply differential redox control during B to plasma cell differentiation. ERAD: ER-associated degradation.

R.S., unpublished observations). Therefore, the failure of B cells to polymerize IgM and IgA is probably due to the differential expression of molecules involved in ER redox control in B and plasma cells.

In a search of molecule(s) interacting with Ero1α, we recently identified ERp44. This protein contains a thioredoxin-like domain with a conserved CRFS motif in the active site, and can form mixed disulfides with both Ero1α and β as well as with unassembled Ig subunits (Anelli *et al.*, 2002, and unpublished observations). Interestingly, ERp44 is one of the proteins whose relative abundance increases most dramatically during terminal B cell differentiation (van Anken *et al.*, 2003). The capability of ERp44 to form mixed disulfides not only with Ero1 proteins, but also with intermediates in oxidative protein folding, including unassembled Ig subunits (Anelli *et al.*, 2002; T.A., A.M. and R.S., unpublished results) suggests a role for this protein in thiol-mediated ER quality control (Fra *et al.*, 1993). It is tempting to speculate that, by retaining μ2L2 subunits and J chains, ERp44 could facilitate IgM polymerization. Indeed, ERp44 could recruit also Ero1 molecules, which would provide the necessary oxidative equivalents. It will be important to identify the molecules that mediate the formation and dissociation of the mixed disulfides between ERp44 and proteins with unpaired cysteines.

The different behavior of B and plasma cells with respect to polymerization, degradation and secretion of IgM suggest that either cell- or stage-specific chaperones and oxidoreductases are expressed or that the relative abundance of some key redox controllers varies during B cell differentiation.

Further studies are needed to understand the fine regulation of the UPR in the biogenesis of professional secretory cells and to identify novel lineage-specific components of the ER folding machinery. The latter may provide useful tools in biotechnology and represent targets for therapeutical intervention to selectively modulate protein secretion and degradation.

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Progressively impaired proteasomal capacity during terminal plasma cell differentiation

Simone Cenci[1,2,7], Alexandre Mezghrani[1,7,8], Paolo Cascio[3,7], Giada Bianchi[1,2], Fulvia Cerruti[3], Anna Fra[4], Hugues Lelouard[5], Silvia Masciarelli[1,2], Laura Mattioli[6], Laura Oliva[1], Andrea Orsi[1,2], Elena Pasqualetto[1], Philippe Pierre[5], Elena Ruffato[1], Luigina Tagliavacca[1] and Roberto Sitia[1,2,*]

[1]Department of Biology and Technology, DiBiT, San Raffaele Scientific Institute, Milan, Italy, [2]Università Vita-Salute San Raffaele, Milan, Italy, [3]Department of Veterinary Morphophysiology, University of Turin, Grugliasco (TO), Italy, [4]Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy, [5]Centre d'Immunologie de Marseille-Luminy, CNRS-INSERM-Université de la Meditérannée, Marseille, France and [6]Department of Experimental Medicine, University of Genoa, Genoa, Italy

After few days of intense immunoglobulin (Ig) secretion, most plasma cells undergo apoptosis, thus ending the humoral immune response. We asked whether intrinsic factors link plasma cell lifespan to Ig secretion. Here we show that in the late phases of plasmacytic differentiation, when antibody production becomes maximal, proteasomal activity decreases. The excessive load for the reduced proteolytic capacity correlates with accumulation of poly-ubiquitinated proteins, stabilization of endogenous proteasomal substrates (including Xbp1s, IκBα, and Bax), onset of apoptosis, and sensitization to proteasome inhibitors (PI). These events can be reproduced by expressing Ig-μ chain in nonlymphoid cells. Our results suggest that a developmental program links plasma cell death to protein production, and help explaining the peculiar sensitivity of normal and malignant plasma cells to PI.

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Introduction

Upon encounter with antigen, B-lymphocytes activate a complex program involving proliferation, generation of memory cells, isotype switch, and affinity maturation. Rapid defense is guaranteed by the differentiation of antibody-secreting plasma cells (Calame *et al*, 2003; Brewer and Hendershot, 2005). Although a minority of them home in the bone marrow and survive for longer periods, most plasma cells are short-lived and succumb after a few days of intense immunoglobulin (Ig) secretion, particularly those producing IgM (Ho *et al*, 1986; Manz *et al*, 2002). Disregulated plasma cell lifespan can cause autoimmune diseases and tumors (Kuehl and Bergsagel, 2002; Hoyer *et al*, 2004). Although it is clear that extrinsic factors are key to determine the proper environment for long-lived plasma cells, rather little is known on the molecular events that cause apoptosis in short-lived plasma cells. In particular, whether intrinsic factors play a role in limiting plasma cell lifespan is undetermined. An intriguing possibility is that cell death is linked to antibody production.

To explore these issues, we exploited murine primary B cells and a B lymphoma (I.29μ$^+$) that can be induced to differentiate into massive IgM secretion (van Anken *et al*, 2003). After lipopolysaccharide (LPS) stimulation, I.29μ$^+$ activate a developmental program that induces the stepwise synthesis of different classes of proteins so as to increase the secretory capacity. Endoplasmic reticulum (ER)-resident proteins increase over the entire time of the experiment, whereas IgM subunits increase significantly after 2 or 3 days, to become the dominant molecular species synthesized in the last days of differentiation (van Anken *et al*, 2003). LPS-stimulated I.29μ$^+$ cells begin to die after day 3 or 4, concomitantly with massive IgM secretion and accumulation of spliced Xbp1 (Xbp1s). This transcription factor is activated during the unfolded protein response (UPR) and is essential for plasma cell development (Reimold *et al*, 2001; Iwakoshi *et al*, 2003b). Xbp1s drives the transcription of genes encoding factors involved in secretion (Shaffer *et al*, 2004; Sriburi *et al*, 2004; Tirosh *et al*, 2005) and ER-associated degradation (ERAD) (Yoshida *et al*, 2003). Promoting the efficiency of protein secretion is of obvious importance for plasma cells, which are specialized in Ig production. Why could ERAD regulation be so important? A considerable fraction of secretory μ-chains are degraded also in hybridoma cells (Fra *et al*, 1993; Fagioli and Sitia, 2001), probably owing to the inefficient assembly of IgM polymers, the only species that negotiate export from the ER (Sitia *et al*, 1990; Cals *et al*, 1996). We reasoned that the exuberant production of IgM that characterizes activated B cells could represent a load on the protein degradation machinery, which is largely dependent on cytosolic proteasomes (Mancini *et al*, 2000; Fagioli *et al*, 2001; Goldberg, 2003). This idea stemmed also from the fact that proteasome inhibitors (PI) proved particularly effective in the therapy of multiple myeloma (MM), by inducing apoptosis of malignant cells (Hideshima *et al*, 2003; Adams, 2004). Therefore, we decided to test a model that, described in broad terms, predicts that apoptosis of short-lived plasma cells and the exquisite sensitivity of plasma cells to PI are promoted by an unbalance between load (protein synthesis) and capacity

*Corresponding author. Department of Cell & Molecular Biology, Università Vita-Salute San Raffaele, DiBiT-HSR, Via Olgettina 58, Milan 20132, Italy. Tel.: +39 02 2643 4722; Fax: +39 02 2643 4723; E-mail: r.sitia@hsr.it
[7]These authors contributed equally to this work
[8]Present address: Institut Genetique Humaine, CNRS Montpellier, France

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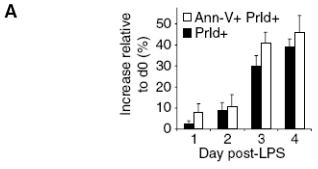
of the proteolytic machinery (proteasomes). Our results suggest that this may be indeed the case. When Ig synthesis becomes maximal, the abundance of proteasomes decreases in both I.29μ^+ and primary B cells. This correlates with reduced proteasome activity, accumulation of polyubiquitinated proteins, enhanced susceptibility to PI, and stabilization of several proteasome substrates important in B-cell physiology (Xbp1s, IκBα, and Bax). Similar events can be reproduced in HeLa cells forced to express μ-chains in large quantities. Hence, we propose that the unbalance between proteasomal load and capacity intrinsically exaggerates ER stress and predisposes plasma cells to apoptosis. These findings help explaining the exquisite sensitivity of normal and malignant plasma cells to PI.

Results

In LPS-activated I.29μ⁺ cells, apoptosis correlates with exuberant IgM production

I.29μ^+ are B lymphoma cells that can differentiate and acquire a plasmacytoid phenotype if stimulated with LPS (van Anken *et al*, 2003). After 3–4 days of LPS treatment, concomitantly with massive IgM synthesis and secretion, I.29μ^+ cells undergo apoptosis, thus mimicking the fate of short-lived plasma cells. Indeed, after 3–4 days of stimulation, the frequency of apoptotic cells increases sharply (Figure 1A). Apoptotic cells contain high amounts of intracellular μ-chains, correlating cell death with IgM production (Figure 1B). Following an initial acceleration in cell division, apoptotic cells accumulated. The onset of apoptosis correlated with a decrease in G2/M cells (Supplementary Figure 1A). LPS-treated I.29μ^+ cells divided more rapidly at day 1 and less at days 3–4 (Supplementary Figure 1B). Caspases 3, 8, and 9 were strongly activated after day 3 (Supplementary Figure 1C).



	R1		R2		R3	
Day	%	MFI	%	MFI	%	MFI
0	85	491	10	1541	4.5	1845
1	76	677	18	1534	6	1373
2	59	981	32	2678	9	1660
3	37	815	38	2874	23	2585
4	27	973	40	4290	33	3767

Figure 1 LPS-stimulated I.29μ^+ cells undergo apoptosis. (**A**) Cell death was assessed in LPS-stimulated I.29μ^+ cells by Annexin V (Ann-V) and propidium iodide (PrId) staining, and expressed as variation with respect to unstimulated cells. Mean±s.d. of three independent experiments. (**B**) Flow cytometry statistics of μ-chain content in LPS-stimulated I.29μ^+ cells. R1, R2 and R3 were enriched in resting, activated and dead cells, respectively (van Anken *et al*, 2003 and our unpublished data). Mean fluorescent intensity (MFI) is indicated.

Decreased proteasomal levels and activity in differentiating I.29μ⁺ cells

In both B and plasma cells, a fraction of secretory and membrane IgM are rapidly degraded (Sitia *et al*, 1987; Fra *et al*, 1993), mostly by cytosolic proteasomes (Mancini *et al*, 2000; Fagioli and Sitia, 2001; Fagioli *et al*, 2001; Rabinovich *et al*, 2002). The existence of mechanisms that adapt proteasome biogenesis to the degradative demand has been postulated (Meiners *et al*, 2003). Therefore, as the massive increase in Ig synthesis induced in I.29μ^+ cells by LPS (van Anken *et al*, 2003) represents an increased proteolytic demand, we expected proteasomes to increase in abundance. Surprisingly instead, two constitutive proteasomal catalytic subunits (Y and X) significantly decreased after LPS stimulation (Figure 2A and B). Also the interferon-γ (IFNγ)-induced subunits LMP2 and MECL-1 (Cascio *et al*, 2001) decreased, albeit to a lesser extent, whereas LMP7 and Z remained essentially constant.

As a functional counterpart of these results, the proteasomal chymotryptic activity, normalized per protein content, decreased significantly at days 3 and 4 (Figure 2C). Proteasome activity fell dramatically also on a per cell basis (Supplementary Figure 2), indicating that plasma cells do not increase the synthesis and/or assembly of proteasomes to match Ig production.

Caspase activation can inhibit proteasomal activity by cleaving subunits of the 19S regulatory particle (Sun *et al*, 2004). The decrease in proteasome activity could thus be a consequence of I.29μ^+ cells undergoing apoptosis. To exclude this possibility, we compared lysates of total or live cells. Despite Ficoll centrifugation efficiently eliminated dead cells (less than 3 and 7% dead cells were present at days 3 and 4), neither the three main proteasomal activities (panels D–F) nor the abundance of individual subunits differed significantly (panel A, compare lanes 3, 4 and 3F, 4F), apart from μ-chains being slightly more abundant in the fraction enriched in live cells (A, second panel from top). Therefore, the reduction of proteasome activity is not caused by cell death (see also below).

Accumulation of polyubiquitinated proteins in differentiated I.29μ⁺

Consistent with the observed decline of proteasomal activity, polyubiquitinated proteins markedly increased in LPS-activated cells, at the expense of the pool of free Ub (Figure 3A).

To further confirm that the accumulation of polyubiquitinated proteins was not a consequence of apoptosis, we exposed I.29μ^+ cells to UV rays or MG132 (Figure 3B, left panel). Despite UV treatment induced more apoptosis (30%) than proteasomal inhibition (23%), polyubiquitinated proteins did not increase significantly in UV-treated cells. Only a modest decrease of proteasome activity was detected upon UV treatment (Figure 3B, right panel). Induction of apoptosis by UV failed to increase polyubiquitinated proteins and to decrease proteasome activity also in day 3 LPS-stimulated I.29μ^+ (not shown). Thus, apoptosis does not *per se* induce the accumulation of polyubiquitinated proteins in differentiating I.29μ^+ cells.

Accumulation of polyubiquinated proteins was visualized by confocal microscopy with specific antibodies well before overt apoptosis (Figure 3C). Numerous fluorescent dots were detected throughout the cytoplasm after day 3. These structures differed from aggresomes (Kopito and Sitia, 2000) and

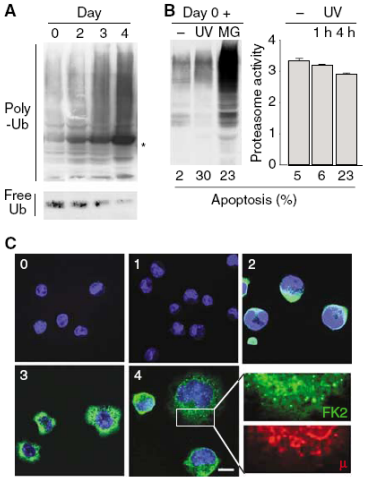


Figure 3 Accumulation of polyubiquitinated proteins in differentiating I.29μ$^+$ cells. (**A**) Extracts of I.29μ$^+$ cells induced with LPS for the indicated times were blotted with anti-Ub. The band indicated with an asterisk consists of μ-chains that crossreact with secondary antibodies. To detect free Ub, a denser gel was employed (bottom panel). (**B**) I.29μ$^+$ cells were treated for 4 h with MG132 (MG) or exposed to ultraviolet light for 35 s and cultured for 4 h (UV, lane 6). In the right panel, proteasome activity was measured in unstimulated I.29μ$^+$ cells cultured for 1 or 4 h after exposure to UV. The percentages of apoptotic cells are indicated at the bottom. (**C**) Immunofluorescent visualization of poly-Ub proteins during I.29μ$^+$ differentiation. Cells were stained with Fk2 antibodies, recognizing poly-Ub proteins, and analyzed by confocal microscopy. Numbers indicate days after LPS stimulation. No overlapping between the Fk2 and anti-μ staining patterns was detected (see a particular at higher magnification in the bottom right panel). Bar: 5 μm.

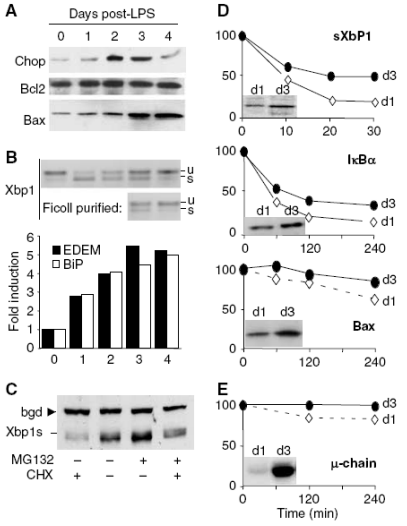


Figure 4 Stabilization of proteasomal substrates in differentiating I.29μ$^+$ cells. (**A**) Extracts from LPS-stimulated I.29μ$^+$ cells were resolved by SDS–PAGE and blots decorated with antibodies specific for Chop, Bcl2, or Bax, as in Figure 2A. (**B**) Xbp1 splicing. The RT–PCR products derived from the spliced (s) and unspliced (u) Xbp1 mRNAs are indicated (top panel). Expression of EDEM1 and BiP mRNAs was measured by quantitative real-time RT–PCR analyses, normalized to actin, and expressed as fold change relative to untreated cells (bottom). (**C**) Xbp1s is degraded by proteasomes. LPS-activated I.29μ$^+$ cells (day 3) were incubated for 20 min with cycloheximide (CHX) and/or MG132, as indicated, lysed and resolved by SDS–PAGE. Blots were decorated with anti-Xbp1. The arrow points to a background stable band (bgd), which served as a loading control. (**D**) I.29μ$^+$ cells, stimulated for 1 or 3 days with LPS, were incubated with CHX for the indicated times. Aliquots of nuclear (IκBα, Xbp1s) or cytosolic (Bax) extracts were resolved by SDS–PAGE and decorated with specific antibodies, as indicated. Relevant bands were quantified by densitometry, and their intensity expressed as the percentage relative to time 0. Inserts show the steady-state protein levels at days 1 and 3. (**E**) I.29μ$^+$ cells treated as above were pulsed for 10 min, chased for the indicated times, and immunoprecipitated with anti-μ. Degradation was calculated by the formula: (intracellular + secreted μ at time *x*): (total μ at time 0) × 100.

concomitantly with the observed onset of apoptosis. To gain further insights into the regulation of UPR components during differentiation, we monitored Xbp1 splicing (Figure 4B, top panel) and the mRNA levels of EDEM (a Xbp1 target gene (Yoshida *et al*, 2003) and BiP (Figure 4B, bottom panel). The efficiency of Xbp1 splicing peaked at day 1, whereas EDEM and BiP transcripts increased in abundance throughout differentiation. The elevated mRNA levels of EDEM at later stages of differentiation—when Xbp1 splicing decreases—could reflect post-translational stabilization of the Xbp1 protein. Indeed, like its unspliced counterpart (Lee *et al*, 2003), Xbp1s is degraded by proteasomes (panel C) and its half-life increases during differentiation (Figure 4D, top panel).

Two other proteasome substrates important for B-cell fate, IκBα (Lin *et al*, 1998) and Bax (Li and Dou, 2000), were stabilized in LPS-stimulated cells (panel D), probably reflecting the decrease in degradative capacity upon differentiation. Ig-μ chains were stabilized as well, as revealed by pulse-chase assays (panel E).

These results indicate that the increased load on the fewer proteasomes causes stabilization of proteins that differ in intracellular localization and intrinsic stability, some of which may facilitate apoptosis.

Increased susceptibility of differentiating I.29μ$^+$ cells to PI

PI are emerging as powerful drugs in MM treatment, but why these tumors are so sensitive is not entirely clear (Hideshima and Anderson, 2002; Goldberg, 2003; Adams, 2004). The above results suggested that the unfavorable load-capacity ratio in Ig-secreting cells could make the latter more prone to enter apoptosis, either basally or following treatment with PI. In this scenario, one would predict that the sensitivity of I.29μ$^+$ cells to PI would increase following LPS stimulation.

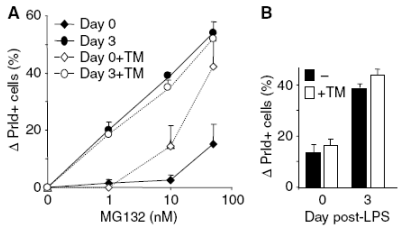


Figure 5 Increased sensitivity to proteasome inhibitors in LPS-stimulated I.29μ$^+$ cells. (**A**) I.29μ$^+$ cells, untreated or stimulated with LPS for 3 days, were cultured for 5 h in the presence of increasing concentrations of MG132, with or without the simultaneous addition of TM (2.5 μg/ml). The percentage of propidium iodide (PrId) positive cells determined by FACS was plotted after subtracting the value obtained without treatment. Mean ± s.d. of three independent experiments. (**B**) TM alone did not significantly induce apoptosis, implying synergy with MG132 in inducing apoptosis in unstimulated I.29μ$^+$ cells.

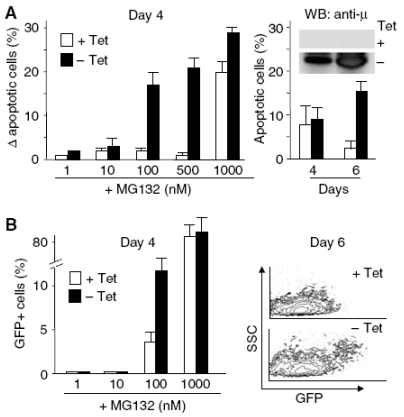


Figure 6 Synthesis of orphan Ig-μ chains sensitizes HeLa cells to proteasome inhibitors and stabilizes a proteasomal reporter. (**A**) Sensitivity to PI increases in HeLa upon Ig-μ synthesis. HeLa-μs Tet-Off cells were cultured with or without tetracycline (Tet) for 2, 4, or 6 days and exposed to increasing concentrations of MG132 for 24 h before FACS analysis. The left panel shows the percentage of apoptotic cells at day 4, after subtracting the values observed without MG132. The right panel shows the basal levels of apoptosis observed at days 4 and 6. The insert shows the accumulation of μs-chains, as revealed by Western blot analyses. At day 2, when little μ-chains accumulated, there were no significant differences in the number of apoptotic cells between induced and noninduced cells (not shown). Data expressed as mean ± s.e.m. of three independent experiments. (**B**) Accumulation of short-lived GFP upon Ig-μ synthesis. HeLa-μs Tet-Off cells stably expressing short-lived GFPs rapidly degraded by proteasomes were generated by lentiviral transduction. As assessed by FACS (left panel), 4 days after Tet removal, GFP accumulated more readily in response to PI (mean ± s.e.m.). After 6 days of intensive Ig-μ synthesis (right panel), some cells displayed spontaneous GFP accumulation by FACS. Axes show side scatter (SSC) on linear scale and GFP fluorescence on log scale.

Therefore, we exposed cells stimulated for 0 or 3 days with LPS to increasing doses of MG132, and determined the fraction of apoptotic cells after 5 h (Figure 5A). Clearly, the sensitivity of LPS-stimulated cells was significantly higher in day 3-stimulated cells. Three additional PI gave similar results (data not shown). To determine whether ER stress could increase the sensitivity to PI, we coincubated cells with tunicamycin (TM), at a dose that did not *per se* induce apoptosis in the experimental time frame utilized (panel B). TM synergized with MG132 in inducing apoptosis in resting, but not in day 3-stimulated cells (panel A), possibly suggesting that the latter were already experiencing ER stress.

Exuberant synthesis of Ig-μ chains makes HeLa cells more sensitive to PI

The above results revealed a correlation between increased Ig-synthesis, decreased proteasomal degradation, ER stress, and apoptosis, both basal and PI-induced. Owing to the complexity of terminal I.29μ$^+$ differentiation (van Anken *et al*, 2003), and to the intrinsic difficulties encountered in transferring genes into B cells, it was not possible to establish causal links. To circumvent this problem and determine whether a cause–effect relationship exists between excessive proteasomal load and apoptosis, we generated stable HeLa cells expressing secretory μ-chains under an inducible promoter (Tet-Off) and analyzed their sensitivity to PI after induction (Figure 6). At day 2, when μ-chain accumulation is still minimal, no differences were detected (data not shown). At day 4, the percentages of apoptotic cells present before the addition of PI were similar in Tet+ or Tet− cells. However, the synthesis of orphan μ-chains clearly made HeLa cells more sensitive to PI (Figure 6A, left panel). The effects of μ-chain synthesis were particularly evident at intermediate doses. After 6 days of induction, μ-chains caused apoptosis in HeLa cells even in the absence of PI (Figure 6A, right panel). Because of the few HeLa-μs Tet-Off cells undergoing apoptosis, single-cell assays were developed based on the expression of short-lived GFPs rapidly degraded by proteasomes as reporters of proteasomal overload (Dantuma *et al*, 2000). After 4 days of μ-chain synthesis, more cells became positive if treated with low doses of PI (Figure 6B, left panel). At day 6, a small yet significant percentage of cells were positive even in the absence of PI (Figure 6B, right panel). Therefore, the synthesis of orphan μ-chains increases the sensitivity of HeLa cells to PI in a time- and dose-dependent way, and eventually causes apoptosis, in correlation with proteasomal overload. Activation of μ-chain synthesis did not affect proteasomal activity (Supplementary Figure 3), indicating that synthetic overload is sufficient to sensitize to PI also in the presence of intact proteasome activity.

Normal differentiating B splenocytes downregulate proteasomes and undergo basal and PI-induced apoptosis

To further generalize our observations, we analyzed primary murine B splenocytes. After LPS stimulation, the relative proteasome capacity fell in the last days of differentiation (Figure 7A), when IgM production becomes maximal (see the Ig-L blot in panel B). Polyubiquitinated proteins accumulated in LPS-activated CD19+ splenocytes, peaking at day 3 (panel

B). Next, to activate B cells by means other than LPS, we cocultured them with CD3-activated T cells (Banchereau *et al*, 1994; Johnson-Leger *et al*, 1998). After an initial rise, proteasomal activity fell dramatically at day 6 (panel C), concomitantly with intracellular accumulation of polyubiquitinated proteins (panel D) and Ig-μ chains (not shown), as revealed by immunofluorescence.

In LPS-activated CD19+ splenocytes, apoptosis increased after day 3 in parallel with mitochondrial damage (panel E), suggesting the involvement of intrinsic pathways. Proteasome inhibition significantly accelerated the death of normal plasma cells, causing apoptosis mainly after day 3 (panel E), again in correlation with the onset of abundant IgM secretion.

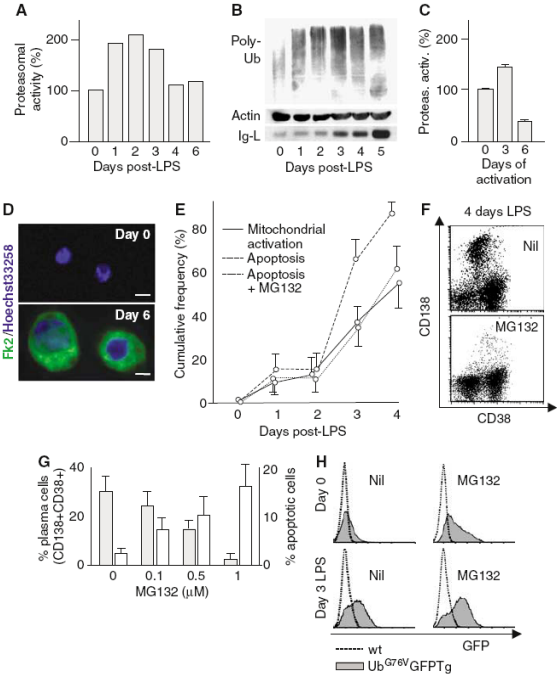


Figure 7 Primary plasma cells accumulate poly-Ub proteins and become sensitive to proteasome inhibitors. (**A**) Proteasomal activity during LPS-induced primary plasma cell differentiation. Proteasomal chymotrypsin-like activity was assessed in extracts from stimulated primary splenocytes as described in legend to Figure 2, and expressed as % relative to day 0. (**B**) Accumulation of poly-Ub proteins in differentiating primary immunomagnetically purified CD19+ cells. Extracts from cells stimulated with LPS for the indicated times were blotted with anti-Ub. Ig light chains (Ig-L) were detected in a denser gel (bottom panel). (**C**) LPS-independent B-cell activation. Unfractionated splenocytes were seeded onto insolubilized anti-CD3 to activate T cells, which in turn cause polyclonal B-cell activation (Banchereau *et al*, 1994; Johnson-Leger *et al*, 1998). At the indicated times CD38+ cells were tested for proteasomal chymotryptic activity as in panel A. (**D**) Cells treated as in (C) were stained with Fk2 antibodies as in legend to Figure 3C. Bar: 5 μm. (**E**) Occurrence of mitochondrial activation (continuous line) and apoptotic death (dotted lines) during LPS-induced plasma cell differentiation from primary B cells. MG132 (100 nM) was added to cultures from day 0. Measurements performed by FACS analysis upon staining with Annexin V and propidium iodide (apoptotic death) or JC-1 (to detect the loss of transmembrane potential across the outer mitochondrial membrane). Mean ± s.d. of three independent experiments. (**F**) FACS profiles of CD38 and CD138 expression in splenocytes 4 days after intraperitoneal injection with 1 mg LPS. Cells were cultured for 6 h in the absence (top panel) or presence of MG132 (bottom panel). (**G**) Proportion of plasma cells (filled bars) and prevalence of apoptosis (empty bars) in splenocytes from *in vivo* LPS-treated mice treated *in vitro* with increasing doses of MG132 for 6 h. Apoptosis was assessed as the proportion of Annexin V-positive cells. Mean ± s.d. (**H**) Accumulation of a short-lived GFP reporter of the Ub–proteasome system in primary B cells from transgenic mice (Lindsten *et al*, 2003) after LPS stimulation. CD19+ cells from Ub^{G76V}GFP transgenic mice and wild-type littermates were immunomagnetically purified and stimulated *in vitro* with LPS for 3 days and with MG132 (1.5 μM for 2.5 h) as indicated.

UV light did not result in significant accumulation of poly-ubiquitinated proteins in apoptotic I.29μ$^+$. The detection of live cells with abundant polyubiquitinated proteins (Figures 4C and 7B) further confirms that in differentiating B cells proteasomal insufficiency precedes apoptosis. In the late stages of differentiation, activated caspases could further decrease proteasomal capacity by cleaving certain 19S subunits, possibly leading to an amplification circuit (Sun *et al*, 2004).

Linking protein production to cell death

A clear cause–effect relationship was established using HeLa cells harboring inducible Ig-μ chains. Overexpression of Ig-μ increased the sensitivity to PI, and resulted in spontaneous apoptosis, similar to what was observed in B cells. Proteasome activity did not decrease in this model, perhaps explaining why the effects were less marked than in activated B cells, where increased load is accompanied by a reduced capacity.

The stabilization of endogenous proteasomal substrates might meet certain functional requirements of plasma cells. For instance, the increased stability of μs chains may favor IgM polymerization, whereas accumulation of death factors may predispose to the apoptotic program. IκBα stabilization would decrease NF-κB activity and lower the apoptotic threshold. Likewise, the stabilization of Bax and Bim, two proteasome substrates known to control B-cell lifespan (Marsden and Strasser, 2003), may—at least in part—explain their relative increase with respect to Bcl2, until the death threshold is reached. In this scenario, plasma cell death would be linked to Ig production, thus contributing to end humoral responses. At the very high rate of Ig production that characterizes the late phases of differentiation, even a small percent of defective degradation would lead to accumulation. Combined with the progressive decrease of proteasomal capacity, this could allow cells to keep track of the amount of Ig secreted.

Generality of the load versus capacity model

The findings obtained with primary B cells indicate that PI hypersensitivity is not a specific feature of myelomas, but is inherent to the processes of plasma cell differentiation and exuberant Ig synthesis, as sensitization can be observed during B to plasma cell differentiation (Figure 7), and artificially reproduced in nonlymphoid cells by overexpressing orphan μ-chains (Figure 6). This has important implications related to the potential utilization of PI in the treatment of inflammatory disorders, especially those related to excessive antibody production. Indeed, epoxomicin inhibits inflammation, and the mechanisms herein described could potentiate NF-κB deregulation (Meng *et al*, 1999; Goldberg and Rock, 2002). With respect to cancer treatment, our findings with I.29μ$^+$ cells suggest that certain lymphomas or leukemias can become sensitive to PI if properly induced to differentiate (Figure 5). In many tumors, factors that induce differentiation facilitate or induce apoptosis (Kasibhatla and Tseng, 2003). Our results suggest that apoptotic sensitivity could be increased by inducing protein synthesis and misfolding. This may sound heretic when degenerative disorders owing to proteotoxicity represent a major social concern. Yet, the observation that TM and PI synergize in I.29μ$^+$ cells provides a proof of principle for utilizing stress against cancer. In the context of plasma cell tumors, the secreting potential of myeloma cells has been proposed as a prognostic factor for MM (Symeonidis *et al*, 2002). Further studies are needed to determine whether PI sensitivity correlates with Ig production and to investigate the features of cytotoxic proteins, with respect to topology, synthesis, folding, or degradation rates.

In principle, the intrinsic 'self-poll**Ub**tion' mechanism described here could operate in other secretory cells in conjunction with diverse extrinsic factors. The latter play a major role in controlling plasma cell lifespan. By providing appropriate soluble factors and costimulatory molecules, germinal centers allow plasmacyte differentiation and survival (Le Bon *et al*, 2001; Manz *et al*, 2002; Poeck *et al*, 2004). Long-lived plasma cells home in the bone marrow, where they find optimal survival conditions, but die when cultured *in vitro*. Based on our findings, one could speculate that long-lived plasma cells reduce their unbalance perhaps by decreasing Ig synthesis, or deploying more degradative capacity, for example, by increasing proteasome biogenesis. Comparing the load-capacity ratios in long- and short-lived plasma cells may shed light into the role of extrinsic and intrinsic life-limiting mechanisms.

In conclusion, we propose that the progressive impairment of proteasomal capacity is one of the mechanisms that contribute in limiting plasma cell lifespan, linking it to antibody production. Identifying the factors that control proteasome synthesis and activity might allow one to manipulate protein production and cell lifespan with obvious implications for biotechnology and cancer therapy.

Materials and methods

Cell cultures and spleen B-cell purification

I.29μ$^+$ cells were activated with LPS as described (van Anken *et al*, 2003). Primary plasma cells were generated from C57Bl/6 mice via three alternative approaches: (1) mice were injected intraperitoneally with 1 mg LPS; after 1–5 days spleen white cells were harvested and assayed for CD38 and CD138 expression and apoptosis by FACS; (2) CD19+ splenocytes from normal or transgenic GFP^{u} mice (Lindsten *et al*, 2003) were prepared by immunomagnetic selection (Miltenyi) and stimulated with LPS *in vitro*; and (3) unfractionated splenocytes were seeded in wells previously coated with anti-mouse CD3 (BD Pharmingen), harvested after 0, 3, or 6 days, and assayed for B-cell content and activation by FACS, proteasomal activity, and immunofluorescent staining for poly-Ub proteins.

HeLa-μs Tet-Off cells were generated by trasfecting Tet-Off cells (Clontech Laboratories Inc.) with pTRE-μ1 and pTK-Hyg. Hygromycin-resistant clones were screened by immunofluorescence after 72 h in the absence of Tet, selected and maintained in medium supplemented with tetracycline. To generate HeLa-μs Tet-Off cells stably expressing the Ub-GFP reporter, lentiviral transduction was adopted (see below and Supplementary data for details).

Flow-cytometric analyses of apoptosis

Cells were washed with PBS and stained with Annexin V–FITC (1 μg/ml) and propidium iodide (2.5 μg/ml). The loss of mitochondrial transmembrane potential across the outer mitochondrial membrane was assessed with JC-1 dye, as described (Bedner *et al*, 1999). Flow cytometry data were obtained with FACScalibur (BD Biosciences) and analyzed using the Cellquest software (BD Biosciences).

Antibodies and immunocytochemistry

I.29μ$^+$ cells were harvested and coated on 1% Alcian blue-treated coverslips for 5 min at 37°C, fixed with 3% paraformaldehyde in PBS for 10 min at RT and permeabilized with 0.5 mg/ml saponin in PBS containing 5% FCS and 10 mM glycine. Primary splenocytes and plasma cells were seeded on poly-L-lysine-coated slides, before

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