Critical Reviews in Biochemistry and Molecular Biology, 40:269–283, 2005

ISSN: 1040-9238 print / 1549-7798 online
DOI: 10.1080/10409230500315352

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Endoplasmic Reticulum Stress and the Making of a Professional Secretory Cell

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ABSTRACT Homeostasis of the protein folding machinery in the endoplasmic reticulum (ER) is maintained via several parallel unfolded protein response pathways that are remarkably conserved from yeast to man. Together, these pathways are integrated into a complex circuitry that can be modulated in various ways, not only to cope with various stress conditions, but also to fine-tune the capacity of the ER folding machinery when precursor cells differentiate into professional secretory cells.

KEYWORDS UPR, ATF6, ATF4, PERK, Ire1, XBP-1, ERAD

INTRODUCTION

The endoplasmic reticulum (ER) is the first compartment of the secretory pathway. It is the cradle of all cell surface proteins, proteins that get secreted and those that reside in any compartment along the exocytic and endocytic pathways. Upon synthesis, they enter the ER lumen via the translocon pore (Johnson & van Waes, 1999; Clemons *et al.*, 2004). In the ER, these proteins fold and oligomerize with assistance of resident chaperones and folding enzymes (Stevens & Argon, 1999). Similar to other folding compartments, the ER harbors chaperones of the Hsp70 family: BiP (Kar2p in yeast) (Hendershot, 2004) and GRP170 (Lhs1p in yeast) (Easton *et al.*, 2000) and a selection of J-domain–containing proteins of the Hsp40 family that serve as co-chaperones for BiP (Hendershot, 2004). Of the Hsp90 family, a single member exists in the mammalian ER, GRP94 (Argon & Simen, 1999), but not in yeast. Also, several representatives from the folding enzyme classes of peptidyl-prolyl *cis-trans* isomerases (PPIases) are present in the ER of both mammals and yeast (Galat, 2003).

Special to the ER is that *N*-linked glycans are added to newly synthesized ER clients and that *N*-linked glycans are important for the folding process in the ER. They facilitate association of the lectin chaperones, calnexin and calreticulin, with folding intermediates to promote their maturation by a mechanism unique to the metazoan ER (Trombetta & Helenius, 1998). Another distinctive element of folding in the ER is the coincident formation of disulfide bonds. Oxidative folding is assisted by ER-specific folding enzymes of the protein disulfide isomerase (PDI) family (Ferrari & Soling, 1999). Oxidized PDI acts as electron acceptor for free sulfhydryl groups and hence as a disulfide donor for client proteins. In the process, PDI is reduced (Freedman *et al.*, 1994). Reduced PDI in turn catalyzes isomerization of disulfide bonds; aberrant disulfide bonds are disentangled in favor of the formation of native disulfide bonds (Freedman

Editor: Michael M. Cox

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et al., 1994). To sustain the net flux of disulfides into proteins that are secreted, however, PDI must be re-oxidized, a process which is the responsibility of the Ero1 protein. As terminal electron acceptor, Ero1 uses molecular oxygen, via FAD (Tu & Weissman, 2004).

The ER chaperones and folding enzymes no longer interact with client proteins after they have fully folded and oligomerized. Instead, mature client proteins exit the ER and travel to their final destination, in or outside the cell. Conversely, the association of ER resident folding factors to incompletely folded or misfolded ER client proteins inherently leads to their retention. This selection procedure based on folding status is referred to as ER quality control (Ellgaard & Helenius, 2003).

Some ER client molecules never reach maturity, because they misfold beyond rescue. Folding failures are recognized as such in the ER lumen, for instance, by the lectin EDEM (Molinari *et al.*, 2003; Oda *et al.*, 2003). Through retrotranslocon pores, folding failures are sent back to the cytosol, where they are submitted to ubiquitination, followed by proteasomal degradation in a similar manner as ill-fated cytosolic proteins (Tsai *et al.*, 2002). This process is referred to as ER associated degradation (ERAD).

Fidelity of folding in the ER may be guaranteed by the efforts of ER quality control and ERAD machineries under basal conditions. To maintain homeostasis, however, the ER needs to adapt to changes in folding load, on both a quantitative and qualitative level. Misfolded mutant ER-clients that accumulate in the ER lumen indeed induce upregulation of ER resident proteins (Kozutsumi *et al.*, 1988). A similar response is provoked by drugs that hamper productive folding in the ER—such as tunicamycin, which interferes with *N*-glycosylation (Mori *et al.*, 1992; Cox *et al.*, 1993); DTT, which blocks disulfide bond formation (Braakman *et al.*, 1992); or thapsigargin, which depletes calcium from the ER (Li *et al.*, 1993). The intricate sensing and response mechanisms that monitor folding load or 'ER stress' are collectively referred to as the unfolded protein response (UPR) pathways. Together, they aim for enhanced folding capacity of the ER on the one hand, and for relief from folding load via ERAD or via a block in protein synthesis on the other (Figure 1).

Over the past few years, it has become increasingly clear that the UPR pathways are remarkably conserved between yeast and man. Perhaps even more exciting is that the UPR pathways appear to orchestrate transformation of precursor cells into professional secretory cells, for instance, during B cell differentiation.

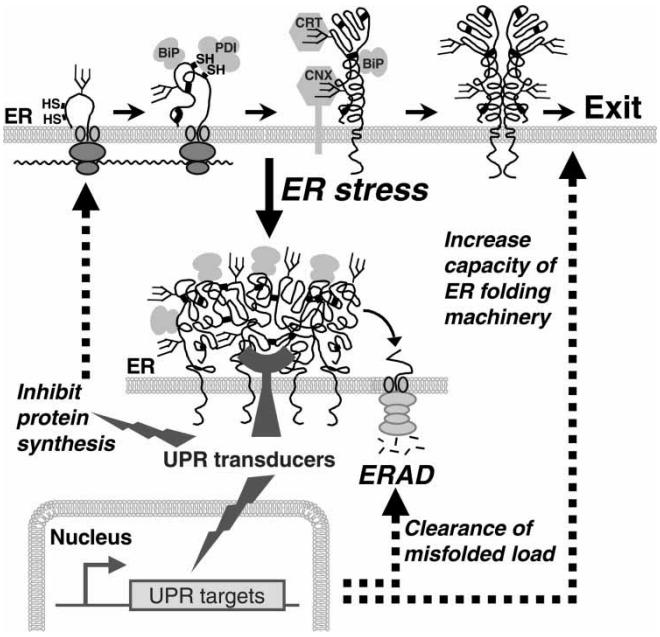

FIGURE 1 **The UPR as instrument for maintenance of ER homeostasis. Proteins destined for the secretory pathway are translocated into the ER, where they obtain *N*-linked glycans and fold and oligomerize with assistance of ER resident chaperones, such as BiP and, in metazoans, calnexin (CNX) and calreticulin (CRT). Folding of ER clients goes hand in hand with disulfide bond formation, a reaction that is catalyzed by PDI or its family members. Only correctly folded and assembled protein products exit from the ER. Thus, when stress is imposed on the ER folding machinery, misfolded proteins accumulate and/or aggregate in the ER lumen. Various UPR 'detectors' sense the presence of accumulated ER load and transduce this signal to the nucleus, where they activate a range of UPR targets. UPR targets aim for enhanced folding capacity of the ER, disposal of folding failures from the ER via ERAD and/or overall cellular stress adaptation. Apart from the UPR gene expression programs, one branch of the metazoan UPR inhibits protein synthesis to relieve the ER folding machinery from further entry of folding load.**

UPR TARGETS

Transcription levels of approximately 400 genes, i.e., 7% to 8% of the yeast genome, are upregulated via the UPR pathways (Travers *et al.*, 2000). Among the UPR targets in yeast are a complete set of ER folding factors, as well as proteins involved in lipid biosynthesis (Travers *et al.*, 2000), reflecting that both the ER membrane and the ER lumenal content expand to accommodate an increase in folding load (Cox *et al.*, 1993; Cox *et al.*, 1997). In addition, many UPR targets represent proteins that have a role in ERAD; (Travers *et al.*, 2000). In fact, a functional UPR is required for efficient ERAD; overexpression of misfolding mutant proteins induce a UPR, which in turn augments transcription of

ERAD components to a level necessary for the clearance of the misfolded mutant proteins from the ER lumen (Casagrande *et al.*, 2000; Friedlander *et al.*, 2000).

The list of mammalian UPR targets is far from exhaustive at present but, as in yeast, includes ER resident folding factors, ERAD components, and enzymes of lipid biosynthesis pathways (Harding *et al.*, 2003; Lee *et al.*, 2003; Shaffer *et al.*, 2004; Sriburi *et al.*, 2004). Moreover, several other functional categories—such as genes encoding mitochondrial proteins, redox balance proteins, or proteins involved in amino acid synthesis and import—are targets of the mammalian UPR (Harding *et al.*, 2003).

UPR PROMOTER ELEMENTS AND TRANSCRIPTION FACTORS

Most genes that encode ER folding factors in yeast contain one or more *cis*-acting unfolded protein response elements (UPRE) in their promoter regions (Mori *et al.*, 1992). Still, only about one sixth of the ~400 UPR targets are regulated via this 'canonical' UPRE (Patil *et al.*, 2004). Two recently identified upstream activating sequences, UPRE-2 and UPRE-3, serve as alternative to the 'canonical' UPRE. Together, they account for the *trans*-activation of another one third of UPR targets (Patil *et al.*, 2004). Yet, for about half of the UPR targets, it remains unclear what *cis*-acting element controls their transcription.

Also in mammals, various upstream activating sequences convey UPR induced transcription (Table 1): the ER stress element (ERSE) (Yoshida *et al.*, 2000; Yoshida *et al.*, 2001) or a variant thereof, ERSE-II (Kokame *et al.*, 2001), the mammalian UPRE (Yoshida *et al.*, 2001), the ATF/CRE *cis*-acting element (Luo *et al.*, 2003; Ma & Hendershot, 2003), and the C/EBP-ATF *cis*-acting element (Ma *et al.*, 2002; Ma & Hendershot, 2004), also known as amino acid response element (Okada *et al.*, 2002). Several other UPR related upstream activating sequences may remain to be identified.

The variety in *cis*-acting elements among UPR targets reflects the variety in *trans*-activators that transduce the UPR. All UPR *trans*-activators identified thus far are members of the family of basic leucine zipper (bZIP) transcription factors: Hac1p and Gcn4p in yeast and ATF6α, XBP-1, and ATF4 in mammals. The bZIP *trans*-activators can form both homo- and heterodimeric complexes. Altogether, the variety in *cis*-acting elements and bZIP transcription factors involved reflect the complexity of the circuitry of the UPR pathways. While many key elements are similar in yeast, the mammalian UPR circuitry is more expanded.

THE Ire1/Hac1/XBP-1 PATHWAY

How are ER stress or an increase in folding load detected and translated into the activation of UPR-specific transcription factors? Conserved from yeast to man is the sensing and response pathway that is transduced by Ire1 (Figure 2). Ire1p in yeast (Cox *et al.*, 1993; Mori *et al.*, 1993) and its mammalian homologs, Ire1 α. (Tirasophon *et al.*, 1998) and Ire1β (Wang *et al.*, 1998), consist of an unfolded protein sensor domain in the ER lumen connected via a transmembrane domain to an effector domain in the cytosol. BiP associates with the lumenal domain of Ire1 under basal conditions (Bertolotti

TABLE 1 **Mammalian UPR promoter elements, transcription factors, and targets**

Promoter element	Sequence consensus	Transcription factor	Targets	References
ERSE	CCAAT-$(N)_9$-CCACG	ATF6α/XBP-1	BiP, CRT, PDI, ERp57, etc.	(Yoshida *et al.*, 1998)/(Okada *et al.*, 2002)
			XBP-1	(Yoshida *et al.*, 2001)
			$p58^{IPK}$	(Yan *et al.*, 2002)
			CHOP	(Ubeda & Habener, 2000)
ERSE-II	ATTGG-N-CCACG	ATF6α/XBP-1	Herp	(Kokame *et al.*, 2001)
mUPRE	$TGACGTG^{G}/_{A}$	XBP-1	EDEM	(Yoshida *et al.*, 2003)
ATF/CRE	$TGACGT^{G}/_{C}A$	ATF4/OASIS	BiP	(Luo *et al.*, 2003; Kondo *et al.*, 2005)
		ATF4	GADD34	(Ma & Hendershot, 2003)
C/EBP-ATF	$^{G}/_{A}TT^{G}/_{T}CATCA$	ATF4	CHOP	(Ma *et al.*, 2002)/(Okada *et al.*, 2002)
			Herp	(Ma & Hendershot, 2004)
			Proteins involved in amino acid metabolism	(Okada *et al.*, 2002)

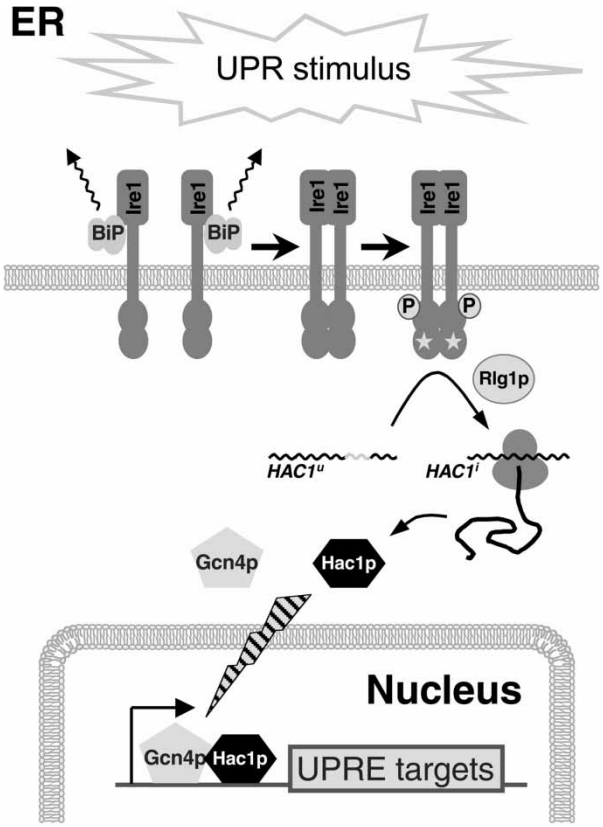

FIGURE 2 **The Ire1/Hac1 pathway in yeast. Central to the UPR in yeast are Ire1p as UPR sensor and Hac1p as UPR transcription factor. The sensing mechanism may involve dissociation of BiP (Kar2p) from the lumenal domain of Ire1p, because the chaperone is recruited by accumulating folding load in the ER. Next, the UPR challenge causes Ire1p to dimerize and *trans*-autophosphorylate. As such, the cytosolic endonuclease effector domain is activated, which in turn mediates splicing of *HAC1*^{u}. Rlg1p religates the transcript to *HAC1*^{i}, which is translated to Hac1p. In the nucleus, Hac1p *trans*-activates UPR targets via various UPRE promoter elements. For the induction of about half the UPR targets, Hac1p needs to team up with Gcn4p. The mammalian homolog of Hac1p is XBP-1. The Ire1 pathway is remarkably conserved between yeast and man, except that a possible connection with the mammalian homolog of Gcn4p, ATF4, still needs to be clarified.**

et al., 2000; Kimata *et al.*, 2003). When unfolded proteins accumulate in the ER lumen, they may compete with the Ire1 proteins for BiP. Hence, dissociation of BiP from Ire1 was proposed to represent Ire1's 'sensing' mechanism (Bertolotti *et al.*, 2000; Kimata *et al.*, 2003). Ire1 lacking the juxtamembrane region that confers BiP binding, however, can still sense and signal ER stress .(Kimata *et al.*, 2004). Thus, activation of Ire1 UPR transducers is not dependent on BiP dissociation *per se*.

When the sensor domain is activated, Ire1 proteins can dimerize and *trans*-autophosphorylate (Shamu & Walter, 1996; Tirasophon *et al.*, 1998). As a consequence, the effector domains on the cytosolic side of the membrane assume endonuclease activity (Sidrauski & Walter, 1997). In yeast, the endonuclease effector domain of Ire1p splices *HAC1* mRNA in a non-canonical fashion (Cox & Walter, 1996). A tRNA ligase, Rlg1p, subsequently religates the *HAC1* transcript (Sidrauski *et al.*, 1996), which is then efficiently translated to the bZIP transcription factor Hac1p (Kawahara *et al.*, 1997). Hac1p travels to the nucleus, where it acts as transcription factor of UPR targets (Cox & Walter, 1996; Mori *et al.*, 1996). For efficient activation of many UPR targets, however, Hac1p needs to team up with its fellow transcription factor Gcn4p (Patil *et al.*, 2004), as will be discussed below.

The X-box binding protein 1 (XBP-1) is the mammalian ortholog of Hac1p, although they share little sequence homology (Shen *et al.*, 2001; Yoshida *et al.*, 2001; Calfon *et al.*, 2002). Translation of the active form of XBP-1, like its yeast counterpart Hac1p, is dependent on Ire1-mediated splicing of its transcript and subsequent religation (Shen *et al.*, 2001; Yoshida *et al.*, 2001; Calfon *et al.*, 2002). The mammalian ortholog of Rlg1p ligase, however, remains to be identified. In the nucleus, XBP-1 can *trans*-activate targets with ERSE or mammalian UPRE upstream activating sequences in conjunction with the general transcription factor NF-Y (Yoshida *et al.*, 2001).

The ATF6 Pathway

A mammalian UPR pathway absent in yeast involves ATF6α (Figure 3). Similar to Ire1, ATF6α is a membrane-spanning protein with an unfolded protein sensor domain in the ER lumen and an effector domain in the cytosol (Yoshida *et al.*, 1998; Haze *et al.*, 1999). Like Ire1, ATF6α may sense accumulation of unfolded proteins in the ER lumen through release of BiP. BiP dissociation unmasks export motifs in ATF6α, which facilitate its travel to the Golgi (Shen *et al.*, 2002). An alternative means of ER stress sensing may be underglycosylation of the lumenal domain of ATF6α. This renders ATF6α a poor substrate for the ER resident lectin chaperones calnexin and/or calreticulin and consequently leads to an 'escape' of ATF6α to the Golgi (Hong *et al.*, 2004).

Upon arrival in the Golgi apparatus, the bZIP effector domain of ATF6α is proteolytically cleaved off by the Site 1 and Site 2 proteases (Ye *et al.*, 2000). Interestingly, these proteases also are responsible for cleavage of sterol regulatory element binding proteins (SREBP) when cholesterol levels drop (Brown &

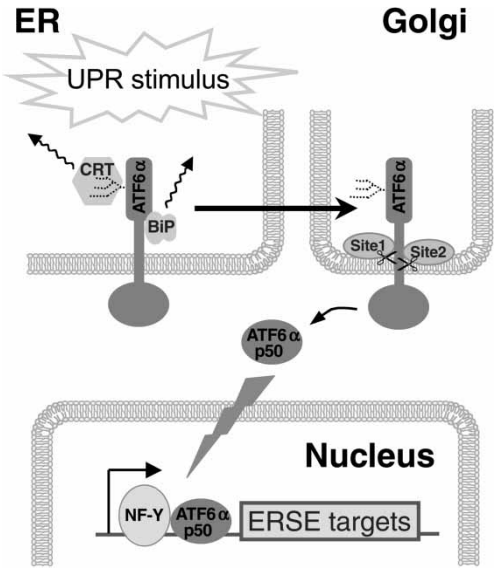

FIGURE 3 **The ATF6 pathway. In metazoans, UPR stimuli can be transduced via ATF6α. Accumulation of folding load in the ER may recruit the chaperone BiP and cause it to dissociate from the lumenal domain of ATF6α. Alternatively, ER stress may lead to underglycosylation of newly synthesized ATF6α (glycan is depicted with dashed lines). As a consequence, ATF6α is no longer recognized by the lectin chaperones like calreticulin (CRT). In either case, ATF6α is no longer retained in the ER through association with chaperones and, hence, it can travel to the Golgi. Upon arrival in the Golgi, proteases Site 1 and Site 2 cleave ATF6α. The cleaved off cytosolic domain of ATF6α (p50) next travels to the nucleus, where it *trans*-activates UPR targets via ERSE promoter elements in conjunction with the general transcription factor NF-Y.**

Goldstein, 1997; Duncan *et al.*, 1998; Sakai *et al.*, 1998). The bZIP effector domains localize to the nucleus, where they can drive transcription of target genes. SREBP activates cholesterol and fatty acid synthesis, whereas cleaved ATF6α (ATF6α-p50) enhances transcription of ERSE targets (Haze *et al.*, 1999) in tandem with the general transcription factor NF-Y (Yoshida *et al.*, 2000).

Two other bZIP family members have an architecture similar to that of the ATF6 proteins and seem to play a role in the UPR pathways. The first is ATF6β (Haze *et al.*, 2001), which may provide negative regulation of ATF6α, as will be discussed below. The second is OASIS. Its signaling mechanism seems to be similar to that of ATF6α, except that the cleaved off bZIP effector domain acts as *trans*-activator of ATF/CRE promoter elements instead of ERSEs (Kondo *et al.*, 2005). The OASIS pathway seems to be particularly important for the UPR in astrocytes, which may relate to the fact that these cells are relatively resistant to ischemia and hypoxia (Kondo *et al.*, 2005).

THE PERK PATHWAY AND THE INTEGRATED STRESS RESPONSE

Both the Ire1/XBP-1 and ATF6α pathways aim for clearance of accumulated load from the ER via reinforced folding capacity. In addition, the Ire1/XBP-1 pathway may relieve the burden on the ER via enhanced ERAD, as will be discussed below. Another means to reduce folding load in the ER is provided by a third mammalian UPR pathway, which is transduced via PERK (Figure 4). PERK has an ER-lumenal domain with homology to that of Ire1 proteins (Harding *et al.*, 1999). Accordingly, PERK may be activated through dissociation from BiP (Bertolotti *et al.*, 2000) and activation entails dimerization and *trans*-autophosphorylation (Harding *et al.*, 1999). Unlike Ire1 proteins, PERK has a cytosolic domain with kinase activity that promotes phosphorylation of initiation factor 2α (eIF2α) (Shi

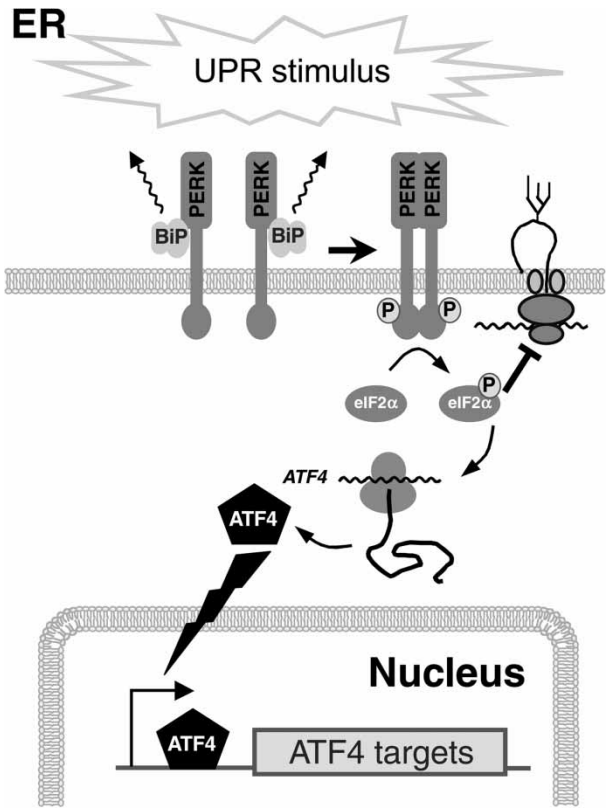

FIGURE 4 **The PERK pathway. In metazoans, UPR stimuli can be transduced via PERK. Like Ire1 and ATF6, PERK may be activated via dissociation of BiP from its lumenal domain. Activation of PERK entails dimerization and *trans*-autophorylation, similar to Ire1. Different from Ire1, the activated cytosolic effector domain of PERK can phosphorylate eIF2α, which in turn inhibits overall protein synthesis. An exception is ATF4. Levels of ATF4 are in fact induced upon phosphorylation of eIF2α. In the nucleus, ATF4 drives transcription of UPR target genes via ATF/CRE or C/EBP-ATF promoter elements.**

et al., 1998; Harding *et al.*, 1999). Because phosphorylated eIF2α blocks translation-initiation, the PERK UPR pathway blocks general protein synthesis. Consequently, numbers of newly synthesized ER clients drop, which may alleviate the burden on the ER folding machinery (Harding *et al.*, 1999; Ron, 2002).

Paradoxically, phosphorylation of eIF2α enhances translation of ATF4 (Harding *et al.*, 2000; Scheuner *et al.*, 2001). ATF4 in turn is a *trans*-activator of UPR target genes (Harding *et al.*, 2000; Novoa *et al.*, 2003). In contrast to ATF6 and XBP-1, ATF4 does not recognize ERSEs. Instead, ATF4 *trans*-activates both C/EBP-ATF (Okada *et al.*, 2002) and ATF/CRE *cis*-acting elements (Luo *et al.*, 2003) in the promoter regions of UPR target genes.

Apart from PERK, several other kinases phosporylate eIF2α in response to, for instance, high levels of reactive metals (Brostrom *et al.*, 1996), the presence of (viral) dsRNA, or amino acid deprivation (Hinnebusch, 1994). Accordingly, all these stress conditions lead to ATF4 signaling. In fact, ATF4 orchestrates a wide range of response mechanisms in addition to the induction of 'classical' UPR targets that aim for homeostasis of the ER folding machinery. For example, ATF4 targets include proteins involved in amino acid metabolism and resistance to oxidative stress (Harding *et al.*, 2003). Since the ATF4 gene expression program has such a central role in cellular homeostasis, it is referred to as the integrated stress response (ISR) (Ron, 2002). Thus far, targets involved in ER homeostasis seem to be regulated by ATF4 via CRE-ATF composite sites, whereas C/EBP-ATF promoter elements seem to correspond to 'overall' stress (Table 1).

Some of the most abundant ER resident proteins were originally identified as glucose regulated proteins (GRPs): BiP (also known as GRP78), GRP94, GRP170, etc. (Shiu *et al.*, 1977). Low glucose levels lead to underglycosylation of ER clients and hence to misfolding. This results in phophorylation of PERK and activation of the ISR (Scheuner *et al.*, 2001). Other signaling pathways next to the ISR may contribute to the induction of GRPs. In a cell line with constitutively low UDP-glucose levels, GRPs are upregulated, but this induction was found to be independent of ERSEs or ATF/CRE *cis*-acting elements (Flores Diaz *et al.*, 2004).

Almost the same set of ER resident proteins were identified independently as oxygen regulated proteins (ORPs) (Heacock & Sutherland, 1986), e.g., GRP170 is also known as ORP150. A decrease in oxygen depletes cellular energy sources and hence induces a drop in UDP-glucose levels. Consequently, hypoxia activates the ISR (Blais *et al.*, 2004). Next to the 'general' ISR, a specific response to hypoxia is transduced in mammals via hypoxia-induced factor 1 (HIF-1). HIF-1 has its own targets distinct from those of ATF4 (Bruick, 2003). Hypoxia directly affects disulfide bond formation and hence protein folding in the ER, since Ero1 uses molecular oxygen as terminal electron acceptor (Tu & Weissman, 2002). Under anaerobic conditions, yeast Ero1p to some extent can use electron acceptors other than oxygen (Tu & Weissman, 2002), but mammalian Ero proteins probably fail to do so. Instead, Ero1α, one of the two mammalian homologs of Ero1p, is under transcriptional control of HIF-1 (Gess *et al.*, 2003).

Interestingly, the O_2-sensor subunit of HIF-1, HIF-1α, localizes under normoxia to distinct 'hotspots' on the perinuclear ER membrane, where reactive oxygen species (ROS) such as hydroxyl radicals are generated in an iron-dependent Fenton reaction (Liu *et al.*, 2004). Only under hypoxic conditions or when this Fenton reaction is inhibited, does HIF-1α migrate to the nucleus, where it acts as transcription factor (Liu *et al.*, 2004). Ero1 is the most likely candidate for the production of these ROS (Tu & Weissman, 2004). Thus, HIF-1 may act as sensor of thiol-oxidation activity in the ER indirectly via levels of ROS generated on the cytosolic side of the ER membrane. As such, it could provide a feedback circuit to adapt Ero1α transcription levels to the overall cellular redox status, apart from the UPR regulation of both Ero1α and the other mammalian Ero1p homolog, Ero1β (Pagani *et al.*, 2000; Gess *et al.*, 2003).

THE ROLE OF Gcn4p IN THE UPR OF YEAST

The mammalian ISR is reminiscent of the yeast general amino acid control. Only a single kinase, Gcn2p, can phosphorylate eIF2α in yeast (Dever *et al.*, 1992), which inhibits protein synthesis but specifically enhances translation of the ATF4 homolog Gcn4p (Hinnebusch, 1997). Gcn4p is a master regulator of gene expression in yeast. At least 539 genes are Gcn4p targets (Natarajan *et al.*, 2001). They encode, among others, enzymes involved in amino acid biosynthesis and autophagy as well as transcription factors,

mitochondrial carrier proteins and peroxisomal components (Natarajan *et al.*, 2001).

Apart from its role in the yeast general amino acid control, Gcn4p appears to be a key element of the UPR (Patil *et al.*, 2004). Together with Hac1p, Gcn4p drives transcription of a subset of UPR targets. To a minor extent, UPRE-2 targets can be *trans*-activated by overexpression of Gcn4p alone, and UPRE-1 targets by high levels of Hac1p alone, but full induction of UPR targets with a UPRE-1, 2, or 3 in their promoter region requires Hac1p to act in tandem with Gcn4p (Patil *et al.*, 2004). In contrast, Gcn4p is not necessary for the regulation of genes without a recognizable UPRE, which represent about half of all UPR targets (Patil *et al.*, 2004). Hac1p alone may be responsible for induction of the last category of UPR targets, forming homodimers or heterodimers with a general, constitutive transcription factor. Alternatively, other as yet unknown UPR-specific transcription factors can team up with Hac1p. Altogether, there is overlap in the targets of Hac1p and Gcn4p, while each transcription factor also has distinct targets, independent from those of the other.

Unlike PERK, Gcn2p lacks a sensor domain in the ER lumen, which would exclude that, in yeast, ER stress is transduced directly to phosphorylation of eIF2α. Nevertheless, yeast lacking Gcn2p cannot mount the integral UPR transcription program, indicating that Gcn2p activity is essential even for basal expression of Gcn4p, and hence for the UPR (Patil *et al.*, 2004). Moreover, Gcn4p levels do rise soon after onset of ER stress (Patil *et al.*, 2004). Perhaps, Gcn4p is stabilized by forming heterodimers with Hac1p (Figure 2), which would account for the rise in Gcn4p levels. Alternatively, Gcn2p is activated upon ER stress in an indirect manner. High doses of tunicamycin indeed lead to phosphorylation of eIF2α in yeast (Cherkasova & Hinnebusch, 2003).

INTEGRATING THE UPR CIRCUITRY

Although levels of Gcn4p increase as a consequence of ER stress, they seem too low to set off the complete yeast general amino acid control program. Instead, Gcn4p is recruited by Hac1p to fine-tune the UPR. Dependent on the duration and severity of the stress(es) that yeast must face, the rise in Gcn4p levels may vary. The UPR gene expression program then would be modulated accordingly, since UPR targets vary in their reliance on Gcn4p for induction (Patil*et al.*, 2004).

In mammals, it remains to be clarified whether ATF4 can form dimers with XBP-1, analogous to Gcn4p and Hac1p in yeast (Figure 2). Along these lines, it is of interest that the mammalian UPRE, which is recognized by XBP-1, and the ATF/CRE composite site, which is recognized by ATF4, are very similar (Wang *et al.*, 2000; Luo *et al.*, 2003; Ma & Hendershot, 2003) (Table 1). If ATF4 and XBP-1 indeed do team up to drive transcription of mammalian UPRE or ATF/CRE composite site targets, ATF4 may regulate the amplitude of induction. This could, in part, explain why UPR target mRNA levels do not correlate linearly with levels of spliced XBP-1 or cleaved ATF6α, (Shang & Lehrman, 2004). It is also possible that other transcription factors can dimerize with and modulate activity of XBP-1, ATF6α or ATF4. For instance, Zhangfei, a poorly characterized bZIP family member, can team up with either ATF4 or XBP-1 (Newman & Keating, 2003).

The *XBP-1* gene itself is also under control of an ERSE (Yoshida *et al.*, 2001). Hence, ATF6α can activate its transcription, providing a positive feed-forward circuit to the UPR (Lee *et al.*, 2002). Reminiscent of this ATF6α/XBP-1 gain control, *HAC1* transcription is enhanced when yeast suffers from dual stress conditions, e.g., the presence of a UPR-provoking agent in combination with heat shock (Leber *et al.*, 2004). As a consequence, Hac1p levels rise further than in the 'regular' UPR (Figure 5). This so-called 'super UPR' (S-UPR) supposedly involves a UPR modulatory factor that drives transcription of *HAC1*, analogous to the ATF6α feed-forward effect on XBP-1 transcription. The existence of this UPR modulatory factor, however, still awaits confirmation (Leber *et al.*, 2004).

In proportion to Hac1p levels, transcription levels of many UPR targets increase more under S-UPR conditions than during a 'regular' UPR. Another class of UPR targets, including transcripts of ER folding factors, however, does not display increased transcription during S-UPR, suggesting that their induction is already saturated at 'regular' UPR conditions. Conversely, a few UPR targets are upregulated to much higher levels by the S-UPR than by the 'regular' UPR (Leber *et al.*, 2004). Analogous to Gcn4p, the proposed UPR modulatory factor therefore seems to control the amplitude of a subset of UPR targets. This category includes *INO1*, which is involved in lipid biosynthesis (Hirsch & Henry, 1986), and *DER1*, encoding the yeast homolog of mammalian Derlin-1, which has been proposed as a component of

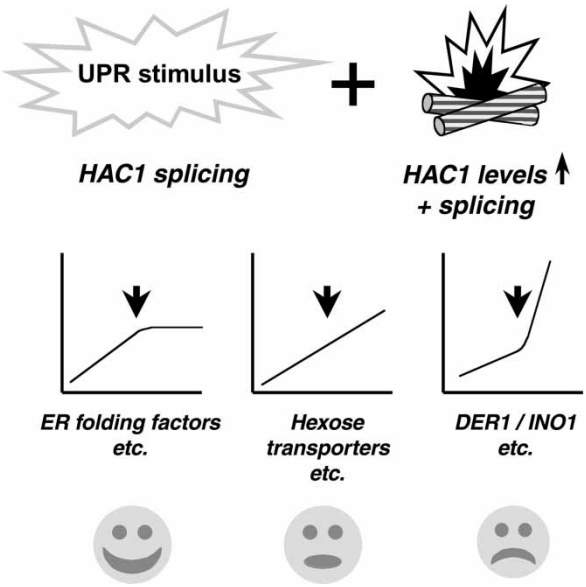

FIGURE 5 **The 'regular' UPR versus the 'super' UPR in yeast. When yeast is challenged with UPR provoking agents, such as tunicamycin or DTT, pre-existing stores of *HAC1* transcripts are spliced, religated and translated into a *trans*-activator of UPR targets. With time, transcript levels of UPR targets rise. When yeast subsequently has to face an additional stress, such as heat shock, levels of *HAC1* transcripts rise, providing positive feed forward on the UPR. This 'super' UPR has variable effects on UPR target induction. ER folding factors do not increase further, while for instance hexose transporters linearly increase after challenge with the additional stress. A third category, representing targets that most likely are dedicated to 'damage control' such as *DER1* and *INO1*, increase to much higher levels under 'super' UPR conditions than under 'regular' UPR conditions. The three classes of UPR targets arbitrarily are categorized as 'optimistic,' 'less optimistic,' and 'pessimistic.'**

the retro-translocon (Lilley & Ploegh, 2004; Ye *et al.*, 2004).

Altogether, it seems that under 'regular' UPR conditions, yeast is still 'optimistic': it upregulates ER folding factors to reinforce folding capacity in the ER and to rescue misfolded proteins. The S-UPR, in contrast, is more 'pessimistic': stress conditions are so severe that yeast more actively expands the ER membrane to accommodate the accumulated ER load, which is considered as misfolded beyond rescue, because the pool of retro-translocons simultaneously increases, most likely to ensure rapid ERAD.

The mammalian UPR shifts from an 'optimistic' to a 'pessimistic' phase in a time-dependent manner (Figure 6). In the first phase, ATF6α is important. Its signaling mechanism is not dependent on *de novo* protein synthesis and is therefore immune to the PERK-mediated translational block. Consistent with the 'optimistic' character of the early phase, the majority of ATF6α targets are ER folding factors (Okada *et al.*, 2002). The Ire1/XBP-1 pathway seems to be invoked later than the ATF6α pathway under sustained ER stress conditions (Yoshida *et al.*, 2001). Most, if not all, ATF6α targets are under control of XBP-1 as well, because both transcription factors *trans*-activate gene expression via ERSE promoter elements. XBP-1 also enhances transcription of UPRE targets. Consequently, the scope of XBP-1 targets is much broader than that of ATF6α. For instance, the ERAD component EDEM is a target of XBP-1, but not of ATF6α (Lee *et al.*, 2003; Yoshida *et al.*, 2003), suggesting that the UPR first aims for rescue of misfolded proteins by enhancing folding capacity via ATF6α. If this attempt is insufficient, the UPR shifts to a 'less optimistic' phase. The Ire1/XBP-1 pathway then is invoked to enhance, in addition, clearance of misfolded load from the ER via ERAD.

ATF6β may play a role when the UPR shifts to a 'pessimistic' phase. The activation and signaling mechanism of ATF6β and ATF6α seem to be identical, except that ATF6β conveys much lower transcriptional activation of ERSE targets (Haze *et al.*, 2001). ATF6β therefore may act as repressor, giving negative feedback on activation of ERSE targets that is mediated by ATF6α (Thuerauf *et al.*, 2004) and possibly XBP-1. Consistent with such a scenario, not a single target could be identified that is dependent on ATF6β for its induction upon ER stress (Lee *et al.*, 2003). More importantly, cleavage of ATF6β occurs later than that of ATF6α (Haze *et al.*, 2001). This finding suggests that when ER stress endures and productive folding in the ER cannot be restored, further attempts to enhance the ER folding capacity are abrogated via ATF6β.

A paradox in the mammalian UPR is that transcripts of UPR targets increase due to the efforts of ATF6α and XBP-1, but that at the same time their translation is inhibited via PERK (Novoa *et al.*, 2001). Since PERK can be phosphorylated early during ER stress, it is crucial that the PERK-mediated translational block can be lifted in due time. One ATF4 target, GADD34, indeed fullfils such a role, because it dephosphorylates eIF2α (Novoa *et al.*, 2001). An alternative negative regulator of PERK is $p58^{IPK}$, which directly inhibits PERK activity (Yan *et al.*, 2002; van Huizen *et al.*, 2003). Interestingly, $p58^{IPK}$ is a target of ATF6α and XBP-1, because its transcription is under control of an ERSE (Yan *et al.*, 2002; van Huizen *et al.*, 2003). Thus, both GADD34 and $p58^{IPK}$ can reconcile the opposing effects of the different branches of the UPR: transcriptional induction and translational

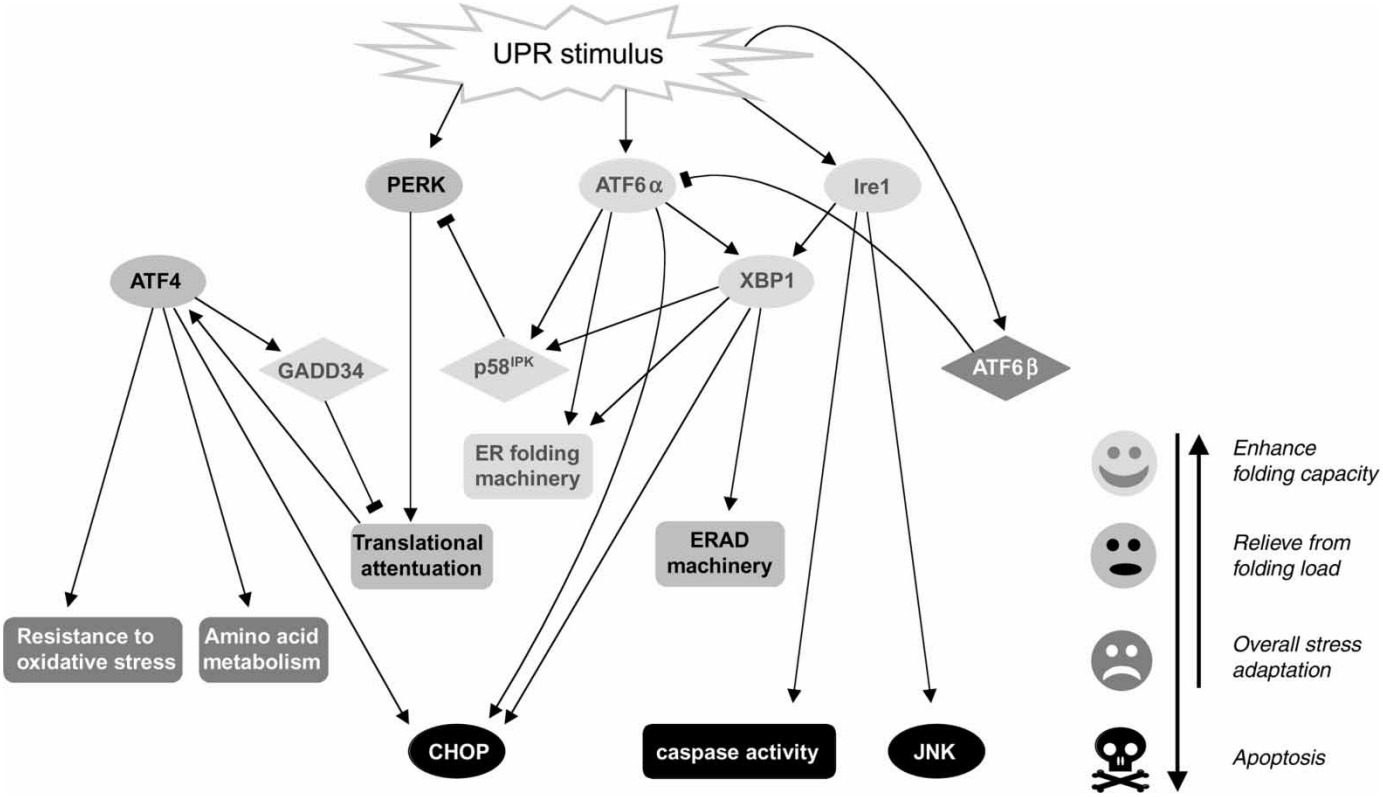

FIGURE 6 **Integrating the UPR circuitry in mammals. A flow chart represents in a simplified manner the various UPR signaling cascades and the way they 'cross-talk.' UPR transducers and transcription factors are depicted as ovals, negative regulators of UPR transducers as diamonds and the effector machineries as rounded rectangles. The various UPR components are coded in gray shades following an arbitrary categorization ranging from 'optimistic' via 'less optimistic' and 'pessimistic' to 'desperate.' Dependent on the nature, duration and magnitude of the UPR stimulus, various components may be engaged in different ways, which determines what the outcome of UPR signaling will be.**

inhibition. As soon as translation resumes, built-up 'stores' of UPR transcripts would ensure that these are preferentially translated. GADD34 indeed is crucial for restoration of translation of UPR targets in fibroblasts (Ma & Hendershot, 2003; Novoa *et al.*, 2003). Still, the 'tug of war' between translation inhibition and transcriptional induction may have variable outcomes dependent on cell type and the challenge imposed on the ER.

ER STRESS AND APOPTOSIS

When ER stress persists and folding problems can no longer be overcome, the UPR shifts to a 'desperate' phase. Prolonged PERK activation mediates cell cycle arrest (Brewer & Diehl, 2000) and cells eventually will die. Accordingly, silencing of $p58^{IPK}$ already leads to apoptosis, because PERK activity is no longer sufficiently counterbalanced (van Huizen *et al.*, 2003). Repetitive but idle attempts to fold ER clients during ER stress involve continued making and breaking of disulfide bonds. As a result, every (re-) oxidation cycle may be accompanied by Ero1-mediated ROS release into the cytosol (Haynes *et al.*, 2004), while elevated cytosolic ROS concentrations lead to apoptosis. The connection between oxidative folding, ROS production, and UPR-induced apoptosis is illustrated further by the finding that decreased expression levels of *pek-1*, the *C. elegans* PERK homolog, lead to reduced viability of the nematode, while concomitant repression of *ero-1* expression to a large extent restores its life span (Harding *et al.*, 2003).

Prolonged ER stress also leads to calcium efflux from the ER lumen into the cytosol (Pahl & Baeuerle, 1997). The rise of calcium levels in mitochondria is an important determinant for UPR-induced caspase activation (Nakagawa & Yuan, 2000) and hence for cell death. The three mammalian UPR pathways share the ability to induce Herp (Ma & Hendershot, 2004). Herp may have an anti-apoptotic role during ER stress, because it seems to stabilize calcium homeostasis, although the mechanism is unknown (Chan *et al.*, 2004).

Herp's efforts can be overruled via pro-apoptotic effectors of the UPR pathways. One of these is the transcription factor CHOP. Like Herp, CHOP is induced via all three arms of the UPR (Kaufman, 2002; Ma *et al.*, 2002). Upregulation of CHOP paves the way to apoptosis (Ron & Habener, 1992; Zinszner *et al.*, 1998), although it is not clear by what mechanism. Ire1α participates in alternative pro-apoptotic pathways. Upon ER stress, the cytosolic domain of Ire1α binds TRAF2, which is an adaptor molecule that couples Ire1 to ASK1. ASK1 in turn can activate JNK, a transcription factor that heralds cell death (Urano *et al.*, 2000; Nishitoh *et al.*, 2002). In addition, TRAF2 can couple Ire1 phosphorylation with caspase-7 and caspase-12 activation (Nakagawa & Yuan, 2000; Rao *et al.*, 2001; Yoneda *et al.*, 2001). The activated caspases again promote cell death via apoptosis.

ER EXPANSION IN PROFESSIONAL SECRETORY CELLS

The reason for the greater complexity of UPR pathways in mammals, as compared to yeast, may be that they allow activation of different subsets of downstream effectors, in particular during development. The Ire/XBP pathway is dispensable for mounting a 'common' UPR in tissue culture cells (Lee *et al.*, 2002), which can be mediated by ATF6α alone (Yoshida *et al.*, 2003). The Ire1 pathways instead seem to be crucial for the development of secretory tissues. Ire1α (Zhang *et al.*, 2005) and XBP-1 (Reimold *et al.*, 2000) are essential for liver development and Ire1β for gut development (Bertolotti *et al.*, 2001).

The most extensively studied example of secretory cell development is the transformation of quiescent B lymphocytes to mature plasma cells. During B cell differentiation, the volume of ER cisternae expands at least threefold (Wiest *et al.*, 1990). Biosynthesis of membranes (Rush *et al.*, 1991) and levels of nearly all known ER resident folding factors significantly increase accordingly (Lewis *et al.*, 1985; Wiest *et al.*, 1990; van Anken *et al.*, 2003). ER expansion is needed to accommodate the bulk biosynthesis of immunoglobulin (Ig). Indicative of the pivotal role the Ire1/XBP-1 UPR pathway plays in physiological ER expansion is the fact that *XBP-1*$^{-/-}$ B cells can only minimally secrete antibody and that transfection of the XBP-1 gene alone can trigger B cell differentiation (Reimold *et al.*, 2001).

Central as XBP-1 is to B cell differentiation, an increase in IgM subunit synthesis and subsequent IgM accumulation in the ER lumen were thought to trigger a 'classical' UPR that could serve as the developmental program driving transformation. However, as evident from *in vitro* differentiation of I.29μ^+ lymphomas (van Anken *et al.*, 2003), B cells carefully prepare for their secretory role as plasma cells (Figure 7). In anticipation of bulk secretion of IgM, they completely reorganize their architecture. B lymphocytes first stock up on mitochondrial and cytosolic chaperones, followed by an increase in metabolic enzymes. Most notably, the ER already expands prior to the onset of high-level IgM production (van Anken *et al.*, 2003). This would exclude

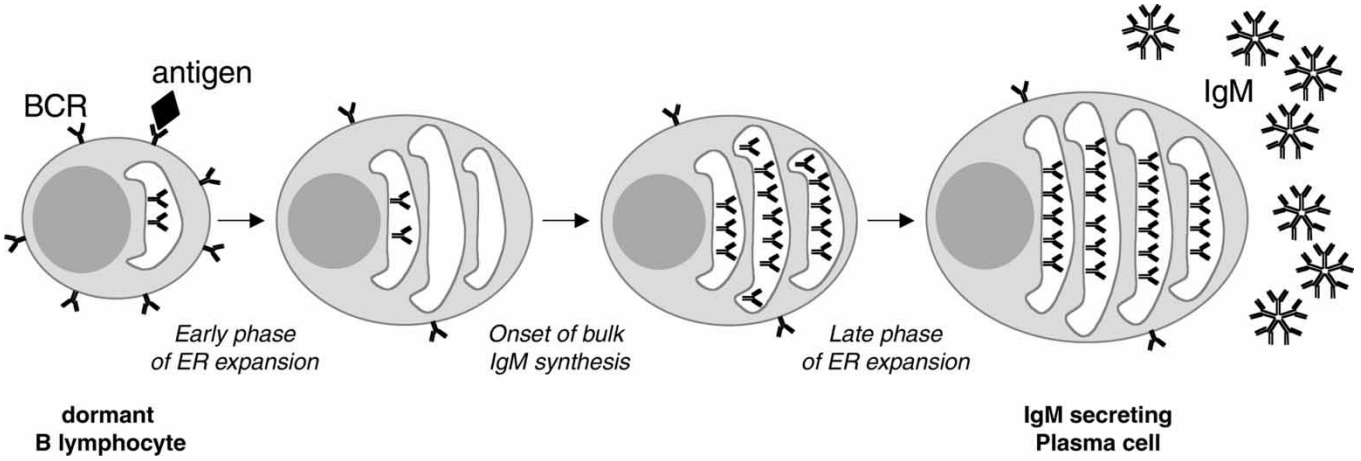

FIGURE 7 **B cells prepare for antibody production. Dormant B lymphocytes express membrane bound IgM as part of a B cell receptor (BCR) complex on their surface. When BCR recognizes and binds a specific antigen (diamond), cells are activated to differentiate into plasma cells. In the early phase of the transformation process, the ER already expands in anticipation of the onset of bulk secretory IgM synthesis that follows. After that, the accumulated IgM subunits in the ER can drive ER expansion further. This two-stage expansion ensures that the ER develops into an efficient 'antibody factory.'**

accumulation of misfolded IgM as the driving force for initial ER expansion. In agreement, during *in vitro* differentiation of CH12 B cell lymphomas, levels of spliced *XBP-1* mRNA already increase prior to the increase in IgM synthesis (Gass *et al.*, 2002), and low levels of *XBP-1* transcripts are spliced upon activation, even when B cells lack the IgM subunit μ heavy chain (Iwakoshi *et al.*, 2003). Only at later stages, when B cells already have considerably enlarged their secretory machinery, is IgM synthesized in such amounts that it accumulates in the ER. The accumulated IgM load may then induce a 'classical' UPR and drive a second phase of ER expansion (van Anken *et al.*, 2003).

If accumulated IgM load is the trigger for late but not for initial ER expansion, then what is? At least three scenarios can be envisaged. One explanation is that the signaling cascades, which emanate from the activated B cell receptor and/or toll-like receptor, can directly intersect with the UPR pathways. There is at present a single precedent for ER-load independent UPR signaling, which involves ATF6α (Xu *et al.*, 2004). Xu and colleagues showed that when intracellular nitric oxide levels are artificially raised to uncouple mitochondrial respiration, calcium leaks out of the mitochondria into the cytosol. The increase of cytosolic calcium levels then activates the Site1 and Site2 proteases, provokes cleavage of ATF6α and, as a consequence, induces transcription of ERSE targets (Xu *et al.*, 2004). How ATF6α translocates from the ER to the Golgi under these conditions remains to be clarified. B cell signaling pathways provoke calcium efflux into the cytosol—not from the mitochondria, but from the ER (Winslow *et al.*, 2003). Calcium release from the ER likewise may be sufficient to set off the ATF6α pathway. Consistent with such a scenario, ATF6α is cleaved early during B cell differentiation, even before production of IgM goes full tilt (Gass *et al.*, 2002).

A second explanation for initial ER expansion is that B cell activation intersects with the UPR pathways via a specific signal in the ER lumen other than accumulated load. Synthesis of secretory IgM rather than membrane-bound IgM is determined by differential splicing of μ heavy-chain transcripts (Sidman, 1981). Early during activation, aberrant splice forms may exist that give rise to μ heavy-chain variants, which could selectively engage UPR sensors. Along these lines, it is of interest that Ire1α seems to be phosphorylated before the onset of massive secretory IgM production (Zhang *et al.*, 2005).

A third explanation for the early ER expansion is that it merely reflects the awakening of B lymphocytes from quiescence before they embark on their IgM secretory mission. In B lymphocytes, XBP-1 transcription is repressed by Pax5, also known as BSAP, (Reimold *et al.*, 1996). Activation of B lymphocytes entails induction of the transcription factor Blimp-1 (Turner *et al.*, 1994), which in turn represses Pax5 (Lin *et al.*, 2002; Shaffer *et al.*, 2002). In other words, Blimp-1 causes derepression of XBP-1 transcription. Thus, XBP-1 transcript levels rise directly as a result of the B cell activation signaling pathways. Basal Ire1 activity would ensure that protein levels of XBP-1 also would increase and hence lead to ER expansion irrespective of accumulated ER load. Transcription of ER folding factors indeed is not induced upon activation of B lymphocytes that lack either XBP-1 or Blimp-1 (Shaffer *et al.*, 2004).

The question remains whether calcium-activated ATF6α, selectively activated Ire1α, de-repressed XBP-1, or a combination of the three orchestrates the early phase of B cell differentiation, but any of these scenarios would imply that the ATF6α/XBP-1 target $p58^{IPK}$ is upregulated well before the onset of bulk IgM synthesis. $P58^{IPK}$ in turn could pre-emptively inhibit PERK, explaining why it does not seem to play a role during B cell differentiation. PERK is not activated in the course of B lymphocyte development into plasma cells (Gass *et al.*, 2002; Zhang *et al.*, 2005). In fact, hematopoietic cells with a defect in eIF2α phosphorylation still can develop into mature IgM secreting B cells, which suggests that PERK activity is dispensable for B cell differentiation (Zhang *et al.*, 2005).

One could argue that overruling PERK-mediated translational inhibition is a logical step in the making of a professional secretory cell. Pancreatic β-cell development, however, exemplifies the contrary. $perk^{-/-}$ mice develop diabetes type I (Harding *et al.*, 2001; Zhang *et al.*, 2002), because unlimited insulin production in β-cells leads to such ER stress that they go into apoptosis (Ron, 2002). How can we explain that β-cells require PERK activity, whereas PERK seems to play no role during B cell differentiation? Perhaps the difference in PERK employment reflects opposing outcomes of an evolutionary 'risk analysis.' As the high prevalence of diabetes type I illustrates, β-cells are particularly vulnerable. The β-cells build up stores of insulin only to be released when demand for this hormone rises. Thus, the UPR pathways must aim for 'sustainable development'

of the β-cell ER. In contrast, plasma cells have a limited life span by default, to give way for Ig class switch, and finally only B cell memory. B cells can therefore more easily run the risk of leaving out PERK, while gaining on secretory capacity.

CONCLUDING REMARKS

Our knowledge of the intimate relations between the UPR pathways and the developmental programs that lie at the basis of the transformation of precursor cells into professional secretory cells has rapidly expanded over the past few years. It has become increasingly clear that there is not a single UPR pathway, but rather that several UPR pathways exist, both in yeast and man. Integration of the different UPR branches varies, depending on the nature, magnitude, and duration of the challenge imposed on the ER folding machinery.

The present challenge is to extrapolate the knowledge of the UPR pathways we gained from ER stress research to the field of secretory cell development. Which UPR pathways are operational in which tissue or cell type? How is the 'tug of war' between translation inhibition and transcriptional induction decided in various cell types? Finally, what is the exact sequence of events during development of various professional secretory cells?

ACKNOWLEDGMENTS

We thank Henk Tabak, Chantal Christis and Viorica Lastun for their valuable comments on the manuscript.

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