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Forum Review

Transmembrane bZIP Transcription Factors in ER Stress Signaling and the Unfolded Protein Response

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ABSTRACT

Regulated intramembrane proteolysis (RIP) of the transmembrane transcription factor ATF6 represents a key step in effecting adaptive response to the presence of unfolded or malfolded protein in the endoplasmic reticulum. Recent studies have highlighted new ATF6-related transmembrane transcription factors. It is likely that current models for ER stress signaling are incomplete and that the expansion of the bZIP transmembrane family reflects selectivity in many aspects of these responses, including the type and duration of any particular stress, the cell type in which it occurs, and the integration with other aspects of cell-type–specific organization or additional intrinsic pathways, and the integration and communication between these pathways, not only in a cell-type–specific manner, but also between different tissues and organs. This review summarizes current information on the bZIP-transmembrane proteins and discusses outstanding questions on the elucidation of the stress signals, the repertoire of components involved in regulating different aspects of the forward transport, cleavage, nuclear import, transcriptional activity, and turnover of each of these factors, and dissection of the integration of the various outputs into broad coordinated responses. *Antioxid. Redox Signal.* 9, 2305–2321.

INTRODUCTION

The endoplasmic reticulum (ER) responds to and regulates many aspects of cellular metabolism and homeostasis. Perhaps one of its most important functions is in providing the site of translation, folding, assembly, and quality control of protein synthesis, encompassing the range of proteins destined for membrane insertion, secretion, and the various locations within the network of intracellular organelles (5, 20, 21). The ER integrates several mechanisms to monitor the load and fidelity of biosynthetic events in protein-export pathways (5, 79). These latter functions are particularly important in tissues with a heavy secretory role, such as immunoglobulin-secreting plasma cells, the pancreas, or the liver.

Numerous general and specific chaperones aid in protein folding and assembly processes (21, 26, 28, 62), and the ER orchestrates quality-control mechanisms whereby misfolded or incompletely assembled proteins are retained to facilitate proper folding, posttranslational modification, and subunit interactions (19, 65). Despite the operation of such mechanisms, failure to achieve the fully folded or assembled product can be a frequent outcome in the normal biosynthetic pathway, and is accentuated during many environmental perturbations or stresses, including nutritional fluctuations, infection, mutation, neoplastic growth, *etc*. In addition to the operation in the ER of cyclic monitoring of folding status, two further key mechanisms regulate the overall levels and balance of functional proteins and the cellular responses to imbalance induced by stress of one form or another. These processes are termed ER-associated degradation (ERAD) and regulated intramembrane proteolysis (RIP), which are components of and integrated with the broad process of the unfolded protein response (UPR).

ERAD is a process that usually controls the degradation of misfolded proteins, but can also be involved in the turnover of

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normal ER-resident proteins (5, 65). Although many mechanistic aspects are still poorly understood, it is generally accepted that ERAD involves the recognition of unfolded or misfolded proteins by ER-resident chaperones such as BiP, calreticulin, and calnexin. This is followed by the unfolding and retrotranslocation through the translocon to redirect the protein out of the ER into the cytosol, a process that is coupled with the ubiquitin-proteasome system on the cytosolic side of the membrane, which aids in translocation and targeting for degradation (5, 63). Recent results highlight the complexity of ERAD wherein different pathways may be involved in the selection and retrotranslocation of unfolded proteins, dependent on whether the unfolded region is within the cytosolic or luminal aspect of the protein (for reviews, see 2, 65, 88).

RIP represents an important overlapping control process in the homeostatic responses to the presence of unfolded proteins, and also in the modulation of fatty acid levels, sterol synthesis, and other ER-associated stresses (6, 30, 110, 115, 117). The key components in these systems comprise a distinct class of membrane-associated transcription factors, anchoring partners that localize the factors to the ER, and proteases that are located in a different compartment. The transcription factors are inserted into ER membranes, with DNA-binding and transcriptional-activation domains oriented toward the cytosolic face of the membrane (30, 34, 96, 107). The main step in controlling the activity of these factors in specific pathways appears to reside in their regulated release from the ER in response to specific stimuli. They are then transported to the Golgi, where they are cleaved in a site-specific manner by resident proteases. This results in the release of the cytosolic domain, which is transported to the nucleus to effect transcription of specific target genes and orchestrate an adaptive response to the stress or imbalance encountered (16, 87, 93, 94).

The prototype members of this class of transcription factors are the sterol regulatory element binding proteins, SREBP 1 and 2, which control genes involved in cholesterol and fatty acid metabolism (35, 96, 110). In response to lower cholesterol levels in ER membranes, SREBPs are transported to the Golgi, where they are cleaved by the Golgi proteases, S1P and S2P (16, 33, 87, 93, 94), thus liberating the SREBP N-terminal transactivation domain of the protein (see later).

This pathway converges with that of quality control of protein folding in the ER with the identification of ATF6, a bZIP transcription factor resident in the ER and also subject to cleavage by S1P and S2P, but in this case, in response to the accumulation of unfolded proteins rather than cholesterol levels (30, 51, 111, 117). ATF6 is one of a triumvirate of signaling components, the others being PERK and IRE1, that recognize the key ER luminal chaperone GRP78/BiP and respond to fluctuations in the ratio of free BiP that are caused by alterations in ER load or the accumulation of unfolded proteins. These three proximal sensors activate interconnected adaptive responses in translation and transcription to counter intracellular stresses resulting from malfolded proteins. More recently, it has become clear that ATF6 is only one of a class of transmembrane bZIP factors that are all involved in integrating adaptive responses. Based on sequence homology to a central section of ATF6 and, in particular, the possession of a bZIP domain adjacent to a transmembrane domain, we and others have demonstrated that certain additional factors are subject to RIP pathways. These factors include Luman/CREB3 (59), OASIS/CREB3L1 (31), CREB4/AIbZIP/CREB3L4 (9, 85), BBF2H7/CREB3L2 (105), and CREB-H/CREB3L3 (81). This review summarizes results on the discovery and role of the prototype bZIP factor ATF6 in UPR and information on the additional new members of this class. It is likely that current models for ER stress signaling are incomplete. Expansion of the bZIP transmembrane family likely reflects selectivity in many aspects of stress signaling, including the type and duration of any particular stress and the cell type in which it occurs. Furthermore, the presence of multiple related sensors may underpin integration of different stress responses with other aspects of cell-type–specific organization or signaling pathways and higher levels of integration and communication between different tissues and organs.

THE PROTOTYPICAL bZIP TRANSCRIPTION FACTOR, ATF6: DNA BINDING AND REFINEMENT OF THE ER STRESS-RESPONSE ELEMENT, ERSE, AND THE UNFOLDED PROTEIN RESPONSE ELEMENT, UPRE

Early insight into the mechanism involved in the UPR stemmed from analyses in yeast using heat shock and glucose depletion to examine the types of responses and the regulatory factors involved (42, 69). Glucose depletion is a physiologically relevant stress that affects glycosylation and energy balance and results in a specific type of adaptive response, including the induction of ER chaperones. Thus, certain ER chaperones also are termed GRPs, for glucose-regulated proteins (40, 49). Using glucose depletion, several UPR-regulated genes, including that for KAR2/BiP, were identified, and a cis-acting element termed the UPRE, encompassing the sequence 5′-CAGCGTG, was found in each of the corresponding promoters (42, 68, 69). The UPRE-binding protein Hac1, a bZIP factor, was subsequently identified and shown to be involved in the ER stress response and transcriptional regulation of selective target genes (13, 67, 77). Although it has been thought that UPR in yeast may be regulated mainly through this motif and by Hac1, more recent analyses indicate that this prototypical UPR may represent only a subset of the Hac1-dependent stress-signaling routes in yeast (84).

Nevertheless, much accumulated evidence indicates that in mammalian cells, regulation of the orthologues of the ER chaperones and GRP genes is considerably more complicated. In particular, studies examining the mammalian orthologue of KAR2 (*i.e.*, GRP78/BiP) identified multiple redundant cis-acting promoter elements involved in the UPR. By analysis of 5′ deletion and linker-scanning mutants of the GRP78 promoter, Lee and co-workers (48) identified a *grp* element, the most important feature of which was a central CCAAT motif. Sequences encompassing this motif and flanking GC-rich elements were able to confer responsiveness to stress inducers, and the motif was subsequently shown to be a binding site for the transcription factor NF-Y/CBF with the flanking region binding YY1 (52–55, 91, 92, 106, 112). Curiously, NF-Y independently actually had a moderate inhibitory effect on the GRP78 promoter

in transfection reconstruction assays, whereas another CCAAT box-binding factor, CTF/NF-1, stimulated expression (112). However, later results from *in vitro* transcription analysis and the use of dominant negative mutants established a positive role for NF-Y in GRP78 transcription (92) and the response to stress inducers (121). Similarly, in transfection assays on cells stressed by a variety of inducers, YY1 was shown to have an augmenting effect on the transcriptional response from the GRP78 promoter (54).

Certain aspects of selectivity in the response of the GRP78 promote were observed. For example, YY1 had little effect on the constitutive activity from the GRP78 promoter, exhibiting an augmentary role only under stressed conditions (54). Similarly, because GRP78 transcription can be regulated by calcium levels, it was shown that NF-Y binding to the CCAAT element was sensitive to calcium levels, offering possible routes to selective transcriptional regulation in response to stress (92). Nevertheless, considering that ubiquitous factors such as NF-Y and YY1 alone may not provide the basis for selective stress-induced transcriptional regulation, the identification of other components was pursued. Subsequent studies refined the nature of the stress-response element within the GRP78 promoter, indicating that the motif actually conformed to the sequence CCAAT-n9-CCACG and that this element was present in multiple copies and conserved in other stress-induced promoters such as those of GRP94 and calreticulin (90, 117). The core and flanking elements originally identified are encompassed within this more-defined motif. The CCAAT-n9-CCACG motif, now termed the ERSE element (for ER stress element) was then used in a one-hybrid screening assay in yeast, where six tandem repeats of the motif were placed upstream of a selectable gene. ERSE-binding proteins were expected to activate expression of the selectable marker, and a clone was then isolated. The resulting clone encoded ATF6, a factor that was first isolated as a member of the ATF class of proteins binding to the core TGACGTAC element (24) and subsequently as a factor that interacts with the serum response factor, SRF (122). ATF6 activates transcription in yeast and in mammalian cells of a target promoter consisting of multiple copies of the ERSE, with both the CCCAAT and CCACG components being required for activation (117). The authors suggested that activation involved NF-Y binding to the CCAAT motif (or in yeast, an alternate CCAAT binding factor such as Hap2-5), combined with ATF6 binding to the CCACG motif of the ERSE (117).

Subsequent studies demonstrated that a cleaved form of ATF6, first observed in stressed cells by Mori and colleagues (117), could form a DNA-binding complex on the ERSE but only in the presence of NF-Y bound to the 5′ CCAAT portion of the element (118). With antibodies to supershift DNA-binding complexes, both NF-Y and ATF6 were observed to be recruited into the DNA-binding complex, with a strict spacing requirement for the location of the CCACG element nine bases from the CCAAT element for DNA binding. NF-Y is itself a heterotrimer of components NF-YA, NF-YB, and NF-YC, and using pull-down assays with individual components, it was shown that ATF6 appears to interact specifically with the NF-YC component of the NF-Y DNA-binding complex (119).

The 3′ motif of the ERSE, CCACG, to which ATF6 binds in the presence of NF-Y, is neither palindromic nor does it resemble the consensus ATF-type site, TGACGTAC, used originally to isolate ATF6 (24) and typical of the central core of bZIP recognition motifs. To examine additional binding activities of ATF6 and any implications for alternative regulatory roles, Wang and colleagues (111) used a DNA-binding site selection and amplification approach to identify a consensus ATF6 binding site (111). The optimal binding site encompassed the sequence (G) (G) TGACGTG$^{G}/_{A}$. This distinct element, termed the UPR element (or UPRE) when inserted in multiple copies, or even as a single copy, upstream of a reporter target, was also shown to confer responsiveness to ER-stress inducers. Mutagenesis of the binding site linked stress responsiveness with ATF6 binding, with the implication that ATF6, in addition to its role on the composite ERSE site, could be involved in ER stress signaling through independent binding (or indeed with different combinations of factors) to the ATF6 site. Although the consensus ATF6 site has been identified in several promoters and in database searches (111), limited definitive evidence exists for a relevant stress-responsive gene regulated by ATF6 through the ATF6 site.

The diversity of cis-acting motifs defining an ER stress-response element is further complicated by the identification of a second version of the ERSE, termed ERSE-II (44). Kokame and colleagues (43) identified a novel gene, Homocysteine-regulated ER Protein, or HERP, whose expression was upregulated by ER stress (43). The promoter for HERP contains a conventional ERSE at around position −90 to −70, and whereas mutation of this element abrogated stress responsiveness, it did not abolish it. A second region at around position −122 encompassing the sequence 5′- ATTGGGCCACG was shown also to play a role in ER stress. The authors proposed that sequence is composed of an inverted CCAAT motif, which would bind NF-Y, now separated by one base (in this case G) from the CCACG motif that binds ATF6. (The two motifs are underlined). Database searching indicated that the ERSE-II element was present in other stress-induced promoters, such as that of ORP150, and that this element may play a role in integrating stress-signaling responses (43). A role of ERSE II in other genes by ATF6 remains to be demonstrated.

ATF6 CLEAVAGE AND ACTIVATION

In the initial identification of ATF6 binding to the ERSE, a cleaved form of the factor was observed specifically in stressed cells (117). These workers went on to characterize stress-induced cleavage of ATF6 using thapsigargin, DTT or tunicamycin. These three agents are used in almost all studies of UPR. Thapsigargin blocks the ER calcium-ATPase pump, thereby depleting the ER calcium store (52). DTT is a strong reducing agent with a broad effect on redox balance, resulting in the reduction of inter- and intraprotein disulfide bonds, the accumulation of unfolded proteins, and their retention in the ER (57). Tunicamycin is a nucleoside antibiotic first observed in *Streptomyces* and inhibits the formation of N-linked glycosylation. Although having clearly quite different mechanisms, a common effect of these agents is the accumulation of unfolded proteins, and they are used as surrogates for more physiologically relevant stress inducers.

With pulse-chase analyses and blotting experiments, thapsi-

gargin, DTT, or tunicamycin induced the appearance of a specific N-terminal cleavage product, p50ATF6 (30). Analysis of ATF6 localization demonstrated that the protein was normally resident in the ER and that it contained a putative transmembrane domain. The p50 cleavage product, conversely, localized to the nucleus, both by fractionation experiments and by immunofluorescence. Comparing the migration of the cleaved p50 product with specific deletion mutants, the authors suggested that ATF6 may be cleaved between the bZIP domain and putative transmembrane domain, but also speculated on the possibility that cleavage may occur within the transmembrane domain, similar to the events involved in cleavage of another transcription factor, SREBP, a transmembrane helix-loop-helix protein central to the regulation of cholesterol regulation (30). Consideration of the regulation of cleavage of SREBP is relevant in the exploration of the role and regulation of the bZIP transmembrane class of proteins

SREBPs contains an N-terminal helix-loop-helix DNA binding and activation domain, a central section containing two transmembrane regions, and a C-terminal regulatory domain (6, 35, 116). This transcription factor is anchored in the ER *via* its two transmembrane domains (6, 96), and when cholesterol levels in the membrane are high, SREBP is retained in the ER in a complex with a polytopic transmembrane protein SCAP (61, 94, 95, 114). Interaction between SCAP and another transmembrane protein, INSIG, is also involved in ER retention of this multiprotein complex (113). When cholesterol levels decrease, a SREBP-SCAP complex is released and is transported to the Golgi, where it is subject to sequential cleavage at two sites (33, 93). The luminal loop is first cleaved at site 1, resulting in a membrane-anchored intermediate. In SREBP2, this site encompasses the sequence, P4-RSVL-P1, and cleavage after the leucine is catalyzed by the site-1 protease, S1P, a membrane-anchored serine protease whose active site is oriented toward the Golgi lumen (16, 94). Cleavage by S1P separates the two transmembrane domains of the SREBPs. The NH_2-terminal product has then a single transmembrane domain and short luminal sequence of 19 amino acids. This intermediate is cleaved in its transmembrane domain by a second protease, the site-2 protease (S2P), a hydrophobic zinc metalloprotease that is tightly embedded in the Golgi membrane. S2P cleaves the SREBP intermediate at the leucine–cysteine bond in the sequence RRSRILLC, where the isoleucine is the first hydrophobic residue of the membrane-spanning segment (17). Although S2P does not show a stringent requirement for particular amino acids at the cleavage site itself, cleavage is influenced by certain specific residues in the DRSR sequence just outside the membrane (17, 33) and by the sequence asparagine-proline (NP), which is located near the middle of the membrane-spanning helix 11 residues to the COOH-terminal side of the cleaved bond. Individual replacement of either the asparagine or the proline reduced cleavage slightly, whereas replacement of both residues abolished cleavage. SCAP is required in the complex with SREBP for recognition and cleavage by S1P. Cleavage by S2P is not thought to be regulated directly by sterols, but is dependent on prior cleavage of SREBP by S1P.

After the demonstration that ATF6 was synthesized as a precursor and cleaved in response to ER stress, Ye and colleagues (115) further demonstrated that ATF6 was transported to the Golgi in response to ER stress induced by DTT (115). They identified an RxxL motif within the ATF6 luminal domain, and an asparagine and proline in its transmembrane domain (although separated by one residue). Using cell lines deficient in S1P or S2P, combined with transfection assays with the ER-localized form of S1P, they showed that ATF6 was subject to cleavage by the same proteases S1P and S2P that cleave SREBP. Interestingly, Although ATF6 cleavage showed complete dependence on S2P, specific cleavage was still observed in cells lacking S1P, prompting speculation that additional proteases other than S1P could be involved (as discussed later).

MECHANISM OF ATF6 RETENTION AND TRANSPORT; BIP THE MASTER REGULATOR

Because S1P and S2P are localized in the Golgi, a key regulatory step requires translocation of ATF6 from the ER to the Golgi (115), a situation clearly similar to that with the SREBPs. Recent analyses have focused on exploring the mechanisms by which ATF6 senses the status of unfolded proteins in the ER lumen, and the subsequent cleavage and processing of ATF6. Early work pointed to the conservation of two regions in the C-terminal luminal domain of ATF6 (115) with the related but still relatively uncharacterized homologue CREB-RP or ATF6β (29, 122). The two conserved domains, referred to as CD1 and CD2 (11), were critical in mediating a transactivation response of a Gal4AD-ATF6 hybrid in response to tunicamycin-induced ER stress but with different mechanistic contributions. CD1, encompassing residues 467–506, was required for translocation to the Golgi after DTT treatment, with internal mutants lacking this region remaining in the ER after treatment. A mutant lacking the CD2 region encompassing the C-terminal residues from 500 to 670 was competent for stress-induced transport, but variants lacking this region or indeed lacking residues 550–670, were not cleaved despite being present in the Golgi. The results indicated a role for this region in regulating S1P cleavage of ATF6 in the Golgi.

Structure–function relations within the luminal domain of ATF6 were refined in subsequent work. ATF6 was observed in a complex with an 80 kDa protein that was not formed by ATF6 mutants unable to traffic to the Golgi in response to DTT treatment (99). This 80 kDa protein, is the cellular chaperone BiP, the central partner in signaling UPR to the other two arms of the UPR, IRE1 and PERK (37). Although deletion analysis indicated that several regions of the ATF6 luminal domain could bind to BiP, the main site of interaction lay within the region between residues 430 and 475 (99). Given its role as an ER-retrieved chaperone, it seemed unlikely that BiP would function as a positive transport signal for ATF6. However, BiP has also been implicated in IRE1 and PERK regulation, in which it is thought to maintain PERK and IRE1α in monomeric inactive states, until ER stress results in BiP dissociation, correlating with activation (4). Although this model has been contested (see later), BiP could therefore function as a repressive signal. Analysis of BiP binding to ATF6 in the presence and absence of DTT demonstrated BiP dissociation on DTT treatment, an event that preceded Golgi transport of ATF6 (99). Furthermore, overexpression of BiP or expression of a variant BiP insensitive to ATP delayed ATF6 transport to the Golgi transport and corre-

spondingly reduced the appearance of cleaved ATF6 species, indicating a direct involvement of BiP in ATF6 ER retention.

Further refinement of the luminal domain identified the presence of two Golgi-localization signals, interdigitated within the BiP binding region, that were required to mediate ATF6 forward transport in response to ER stress. These regions, termed GLS1 and GLS2, mapped between adjacent residues 468–475 and 476–500 within a region termed LD2 (luminal domain 2). By analyzing the trafficking status of various terminal and internal deletion mutants, it was shown that BiP binding suppressed function of the overlapping Golgi-localization signals. The model proposed is that on ER stress, BiP dissociation permits the forward transport of ATF6, mediated by its now active unmasked Golgi-localization signal (99). Importantly, BiP binding *per se* was not responsible for ER retention of ATF6, because in the absence of the GLS motifs, loss of BiP binding was not sufficient to promote forward transport (99).

BiP could regulate ATF6 transport by a number of mechanisms, not mutually exclusive. The first model, a competition model, was based on the assumption that, in the absence of stress, the BiP-ATF6 complex is highly dynamic, with ER stress generating an excess of misfolded substrates that essentially compete with ATF6 for BiP binding. The combination of continuous on–off association of BiP and ATF6, combined with an increase in the accumulation of unfolded proteins to which BiP binds, would result, simply by mass action, in the increase of BiP-free ATF6, the consequential unmasking of the forward GLS motifs in ATF6, and subsequent transport and cleavage. An alternative mechanism was proposed whereby, in the absence of stress, the BiP-ATF6 complex was relatively stable and required active dissociation through a signal actively induced by misfolded proteins. Recent evidence now indicates that, unlike chaperone binding to unfolded proteins, which is typically unstable, BiP binding to ATF6 is comparatively stable. Importantly, the stable BiP–ATF6 complex is actively disrupted in response to ER stress induced by DTT. Although other stable BiP substrates are known, such as Ig heavy chain, BiP dissociation from these substrates was resistant to DTT-induced ER stress. Despite the normal stable interaction of BiP with ATF6, dissociation during ER stress was rapid and efficient, suggesting an active and specific mechanism triggering BiP release (101). These results indicate a revised model in which, in the absence of overt stress, ATF6 is firstly recognized as an intrinsically unfolded protein and is bound by BiP at one or more sites, but specifically within the 430–475 LD2 region. The nature of the BiP binding is, however, qualitatively different from BiP recognition of many unfolded proteins, in that it is an unusually stable one. Within the 430–475 region, a specific ER stress-sensing region exists, which by some mechanism (*e.g.*, through co-chaperone interactions or conformational changes) recognizes the presence of stress, and, as a result, induces active dissociation of BiP from the ATF6 binding sites. This dissociation then results in unmasking of the ATF6 GLS motifs, as indicated earlier. BiP binding is ultimately responsible for ER retention of ATF6, by virtue of the combination of stable binding and masking of the GLS motifs. The proposed mechanism for regulation of ATF6 retention and transport, together with other features discussed in the text, is summarized in Fig. 1.

Finally, it is noteworthy that a soluble form of the ATF6 luminal domain, expressed and extracted in the absence of de-

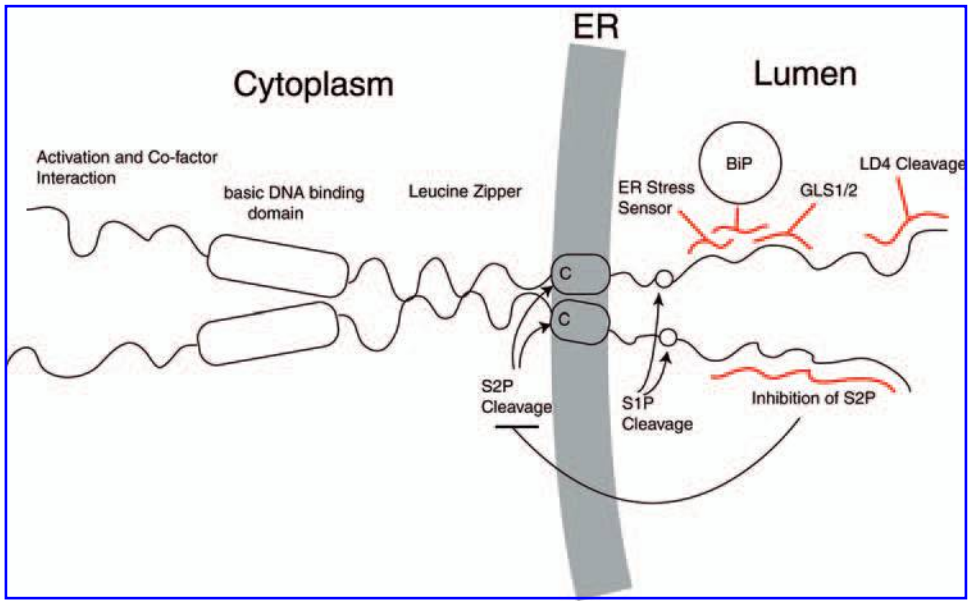

FIG 1. Schematic summary of the organization of ATF6, which comprises an N-terminal activation, DNA binding, and leucine-zipper domains, oriented toward the cytoplasm, followed by a transmembrane domain and a complex luminal domain. The luminal domain encompasses overlapping regions involved in forward transport to the Golgi (GLS1 and 2), BiP-binding regions, and a region that senses ER stress with consequent BiP dissociation and unmasking of the GLS motifs. The distal region of the luminal tail has been shown to be involved in promoting S1P cleavage in the Golgi, whereas the overall length of the region suppresses S2P cleavage at the transmembrane. ATF6 is shown as a dimer, although the nature of homo- and heterdimerization within the membrane is not understood. Studies of ATF6 regulation are discussed and cited in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

tergent, was present in a stable complex with BiP that was resistant to ATP-induced dissociation *in vitro*. When isolated in the presence of detergents, the complex readily dissociated. These results suggested two further aspects of the ATF6–BiP interaction. First, it was proposed that the stability of the complex may be a result of stalling the ATPase cycle of ATF6-bound BiP *in vivo* and that BiP is maintained, in its slow-dissociating ADP-bound form. Second, the results highlighted the role of additional as yet unidentified factors in modulating BiP binding, either through stabilizing the BiP–ATF6 interaction, or by modulating BiP ATP exchange (101). Possible candidates were suggested, including co-chaperone ER homologues of DnaJ, or proteins involved in nucleotide exchange similar to those regulating the cytosolic chaperone, Hsp70.

These results notwithstanding, the precise role and mechanism of BiP involvement in signaling the presence of unfolded proteins remain unclear and, at least for IRE1*a*, recent results have challenged previously accepted models. Mutational analysis of IRE1*a* suggests that the BiP binding region is not essential for IRE1 regulation by the presence of unfolded proteins (41). Furthermore, in recent studies of yeast IRE1α, a conserved core luminal domain (cLD) was identified, and its structure solved to 3.0 Å resolution (14). The cLD (~340 residues) was sufficient to sense the UPR. Structural studies indicate that by virtue of dimerization across a large interface, a groove is created that resembles the gross architectural features of the peptide-binding domain of the major histocompatibility complexes. Mutational analyses indicate that residues that face into the groove are important for UPR activation, and in a distinct revision to previous models, the authors suggest that the cLD directly binds unfolded protein chains rather than BiP (14). In this model, binding of unfolded regions then induces higher-order oligomers of IRE1, and the formation of these oligomers is specifically required to activate the autophosphorylation and RNAse activity of IRE1. Based on conservation within the luminal domains, the same mechanism may apply to PERK. In this revised model, BiP is assigned a secondary role in modulating or buffering the activation response (14). This mechanism for IRE1 activation (and potentially PERK) represents a different route from that now proposed for ATF6, which is itself based on earlier proposals for IRE1.

A FAMILY OF bZIP FACTORS CONTAINING A CONSERVED MULTICOMPONENT DOMAIN SUBJECT TO RIP

Although clearly compelling data exist for a role for ATF6 in mediating the UPR, the identification of other co-factors in modulating its activity will aid in clarifying the precise mechanism. In addition, the growing body of data on new ATF6-related family members is both aiding our understanding of the role and regulation of stress-sensing mechanisms and raising new questions and challenges in understanding the mechanism and the overlap and integration of the factors and pathways involved.

Early work by ourselves and others identified a new protein related to ATF6, known as Luman or CREB3 (59), the murine homologue of which was termed LZIP (7, 8). CREB3/Luman was identified in a screen for cellular binding partners of the human host-cell factor (HCF, also known as C1 factor), a host protein involved in transcriptional activation by the herpes simplex virus transactivator, VP16. Additional related factors were subsequently identified. These factors have various names but are systematically identified under the term CREB3Lx (Creb3-like number). CREB4 (CREB3L4) was originally identified as part of a sequencing analysis of human cDNAs (9). The same protein, termed AIbZIP, was identified in a screen for genes increased in androgen-treated prostate cancer cell lines (85). The factor termed OASIS (old astrocyte specifically induced substance) was identified in mice based on its increase in long-term *versus* short-term cultured astrocytes (31, 80). OASIS is termed CREB3L1. Random sequencing of cDNA clones from the hepatoma cell line Hep2G (81) identified the factor termed CREB-H (CREB3L3). The final member of the family, termed BBF2H7 (CREB3L2), was first identified by virtue of its translocation with the *FUS* gene in a soft-tissue sarcoma (105). Table 1 summarizes the nomenclature of the CREB3 family, their origin, and the potential mammalian homologues.

Although the presence of bZIP region in these proteins was noted in their original identification, consideration of specific features within this region is informative in the broader context of their potential involvement in UPR and the mechanism of RIP. The transcription factors are of a generally similar size, whereas ATF6 is significantly larger (Fig. 2). They exhibit short pockets of limited homology within the N-terminal and C-terminal regions, but the most notable feature is clearly the presence of a highly conserved section, approximately in the middle of each of the proteins. This central region (150 residues), comprises five distinct subregions that, for ease of comparison, are indicated by colored boxes and shown in detail in corresponding shading in Fig. 3.

The basic domain and leucine zipper homologies are labelled as domains II and III, respectively. These regions are extremely well conserved. Each of the factors contains the invariant asparagine and adjacent hydrophobic residues within the core basic domain (Fig. 3, indicated by asterisks), together with the conserved leucine residues at the appropriate spacing (Fig. 3, numbered) which are the defining features of bZIP transcription factors (109). Interestingly, immediately adjacent to the N-terminal end of the bZIP region, an additional region of ~30 residues is found (Fig. 3, region I), which is conserved in the proteins CREB4, Luman, CREB-H, and OASIS, but lacking in ATF6. This latter region is not part of the typical consensus bZIP DNA-binding domain and therefore may provide a distinct function in these factors. Each of these bZIP proteins has been shown to be anchored in the ER through the possession of a hydrophobic transmembrane domain (12, 45, 58, 81, 86, 104). This region (IV), positioned just C-terminal of the leucine zipper, is clearly well conserved in all the factors, and notably contains a completely conserved cysteine residue followed by a run of exclusively hydrophobic residues (see later). Adjacent to this region on the C-terminal side is the next conserved region (region V), which conforms to the consensus site for S1P cleavage (16, 98, 108). Interestingly, inspection of the alignment of the subfamily members (Fig. 3) demonstrates that, apart from ATF6, the motifs conform to the refined consensus S R x $^{L}/_{I}$ x, where R would be the invariant P4 position and P1 showing a strong preference for L. Therefore, it may be that the site represents a specific subset of S1P sites, reflecting some common feature of regulation or cleavage (104).

TABLE 1. SUMMARY OF NOMENCLATURE AND FEATURES OF THE bZIP TM FAMILY

Gene name[a]	*Alternate names (refs.)*	*Genbank accession no. human (mouse)*	*Chromosomal location*[b]	*Full length product*[b]		*Nuclear product*[b]	
				Length (aa)	*MW, kda predicted*	*Length (aa)*	*Mw, kda predicted (apparent)*
CREB3	Luman (59) LZIP (mouse) (7)	NM_006368 (NM_013497)	9p13.3	371	41.4 (55)	229	26 (35)
CREB3L1	OASIS (31, 76)	NM_052854 (NM_011957)	11q11	519	57 (80)	374	41 (50)
CREB3L2	BBF2H7 (46, 105)	NM_194071 (NM_178661)	7q34	519	57.4 (85)	379	42 (60)
CREB3L3	CREB-H (12, 81), CREBh (120)	NM_032607 (NM_032607)	19p13.3	461	49 (76)	322	35 (45)
CREB3L4	CREB4 (9, 104), AlbZIP (85), ATCE1 (mouse) (103), Tisp40 (mouse) (71)	NM_130898 (NM_030080)	1q21.2	395	43.4 (53)	296	32.5 (45)
ATF6α	ATF6 (30, 117, 122)	NM_07348 (AK036335)	1q22-q23	670	74.6 (90)	379	41.3 (60)
CREBL1	CREB-RP (66), ATF6β (29), G13 (39)	NM_004381 (NM_017406)	6p21.3	703	77 (110)	398	42.8 (60)

[a]HUGO Gene Nomenclature Committee name (http://www.gene.ucl.ac.uk/nomenclature/).
[b]Refers to human species.

Although the high degree of conservation in this central domain with ATF6 indicates that these factors are all likely to play a role in sensing ER stress and UPR, it is noteworthy that, outside of the conserved central domain, little overall general homology exists. Moreover, considering the extensive and complex luminal motifs in ATF6 described earlier, all other family members have significantly shorter luminal domains with little homology to each other or to ATF6, suggesting that they could have other roles in ER stress regulation or function in qualitatively distinct ways.

LUMAN/CREB3L3

Luman was identified in a screen for HCF-interacting proteins, an interaction that appears to be mimicked by the herpes simplex virus-1 protein VP16 (58-60). Luman mRNA appears to be expressed in a broad range of tissue types, and the protein product, as examined in transfection assays, is glycosylated (86) and migrates at ~55 kDa. Luman was initially demonstrated to be a member of the RIP family of protein, in that it was subject to proteolysis as a result of treatment with Brefeldin A (86), and it could be cleaved by an ER-localized form of S1P. Mutagenesis of the putative S1P site prevented BFA-mediated cleavage, and subsequent transactivation providing further evidence that Luman was indeed an RIP family member. However, in contrast to ATF6, Luman transport and cleavage was not induced by agents, including tunicamycin, dithiothreitol (DTT), and thapsigargin, that typically induce a UPR response (11, 15, 86). More recently, it was reported that Luman cleavage and activation may be induced in response to thapsigargin (56), a drug that blocks the ER calcium-ATPase pump, thereby depleting the ER calcium store (52). Similar variability has been noted for tunicamycin and DTT treatment of ATF6 (111).

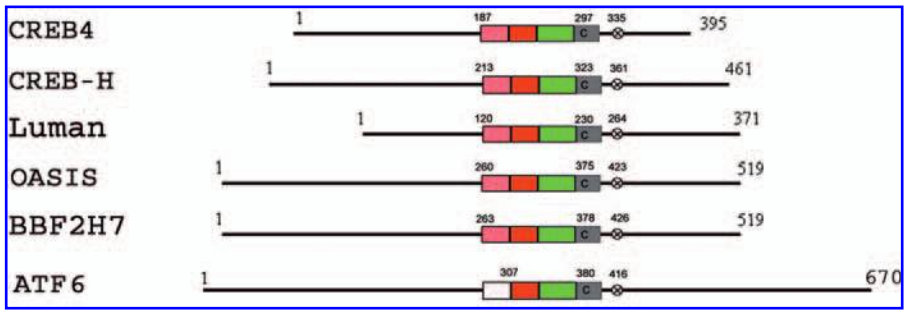

FIG. 2. Comparison of bZIP transcription factors containing transmembrane domains. Schematic illustration of the relative sizes and organization of the central conserved domain in Luman, CREB4, OASIS, CREB-H BBF2H7, and ATF6. The total length of the proteins is indicated, together with the positions of the beginning of the core region of homology, the conserved cysteine within the transmembrane domain, and the arginine of the S1P site. The core region is divided into subregions and colored for ease of reference, as discussed in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

Although the exact cellular function of Luman remains unclear, several studies have been directed at examining its transcriptional properties, promoter specificity, and DNA binding. Luman bound to cyclic AMP response elements (CREs) and C/EBP sites but not to AP-1 sites *in vitro* (59). In contrast, the mouse homologue of Luman, LZIP, was reported to bind to both CRE and AP-1 sites, although the reasons for the difference may be more to do with experimental differences than real differences in DNA-binding properties (7, 86).

Luman activates transcription from artificial promoters containing a CRE site, with activation synergistically enhanced in the presence of a C/EBP element (59). Luman also activates the native promoter for the ER stress-responsive gene EDEM *via* the UPRE consensus element (15), and more recently through the ERSE-II found in a number of genes, including HERP or MIF1 (44, 56). HERP and EDEM proteins are involved in aspects of ER-associated degradation (ERAD), and Luman had a protective survival effect against apoptosis induced through ER stress, providing strong support that Luman has an adaptive response to perturbations in this pathway (56). Interestingly lack of binding to the prototype ERSE suggests that BiP/GRP78 promoter is not activated by Luman, perhaps invoking a role for Luman in other aspects of UPR, such as ERAD (56). Although the relevance is unclear, Luman has also been implicated in cellular transformation, because of its interaction with the HCV core protein, whereby the viral core protein sequesters Luman in the cytosol and potentiates cellular transformation by a mechanism not yet understood (36).

OASIS/CREB3L2

OASIS (CREB3L2) was initially identified as a result of a differential display screen of genes expressed in gliosis, a response to inflammation and trauma within the CNS (31). OASIS was upregulated in long-term cultured astrocytes, and therefore named old astrocyte specifically-induced substance (OASIS). Analyses of the tissue distribution of OASIS expression have yielded somewhat disparate results. In mice, OASIS mRNA was initially reported to be developmentally regulated, being primarily expressed in salivary glands and cartilage in mouse embryos, as detected by *in situ* hybridization. By Northern blot of limited murine tissues samples, expression was seen in lung and kidney in adult tissue. In a separate study of expression during murine embryogenesis, high-level OASIS expression was observed primarily in the skeletal system, including bone-forming tissues and tooth buds (76). Upregulation of expression was also reported in response to injury in the cerebral cortex (31) and spinal cord in adult mice (75). In contrast, analysis of expression of the human gene by Northern blot indicated that human OASIS was expressed mainly in the pancreas but also in the prostate, with lower levels in ovary and colon, and little in the brain (80). Further clarification of the tissue specificity will be informative in understanding its function and, in particular, any qualitative difference in humans *versus* mice.

OASIS is a glycosylated protein that migrates at ~80 kDa and is subject to regulated intramembrane proteolysis by S1P and S2P, respectively. Proteolytic processing generates an ~50-kDa nuclear product (45, 70, 80). OASIS was found to be responsive to the agents tunicamycin and thapsigargin that induce ER stress, resulting in the generation of a cleaved N-terminal product, with corresponding nuclear translocation (45, 70). OASIS expression was also induced in response to ER stress (45), suggesting that it lies downstream or functions toward the late phase of the UPR response. The protein binds to promoters containing B-Box–like elements (80), and in addition to the BiP promoter, which contains both CRE and ERSE sequences (117). However, mutational analyses indicated that the main element by which OASIS acts is the CRE element rather than the ERSE element. Activation of the BiP promoter by OASIS was only modestly affected by deletion of all three ERSE elements, and OASIS was shown to bind CRE elements in bandshift assays, whereas no binding was detected to the ERSE (45). OASIS siRNA knockdown experiments have indicated that OASIS contributes to the induction of BiP mRNA during ER stress, although no effect on the promoters of other UPR responders such as GRP94 or CHOP was observed. With siRNA knockdown experiments, OASIS was also shown to have a protective effect against cell death as a result of induced ER stress (45). This study supplied strong evidence that OASIS was able to activate transcription from the BiP promoter, which correlated with a protective response in astrocytes in response to ER stress.

Details on the mechanism by which OASIS senses stress currently remain unknown. One potentially important feature is the observation that, although it possesses a consensus S1P cleavage site, and that site is cleaved by S1P during ER stress, nevertheless, unlike that of ATF6, S1P cleavage is not necessary for S2P cleavage or for nuclear transport (70). Furthermore, although OASIS binds to BiP, the interaction was not one subject to stress-induced dissociation, in contrast to the ATF6-BiP interaction (70). Although this difference may be due to experimental differences, it may also indicate that the specific signalling mechanism is distinct. Finally, deletion mutants in the C-terminal tail of OASIS retained the ability to undergo stress-induced ER-Golgi transport, prompting the authors to conclude that OASIS did not contain Golgi localization signals comparable to ATF6 (70). It is noteworthy that OASIS was reported to respond to injury and inflammation (31, 75). Inflammation has also been linked to the activity and function of a third member of this family of transcription factors, CREB-H.

CREB-H/CREB3L3

Human CREB-H was identified in a screen for liver-specific transcription factors (81) and in Northern blot analysis of multiple human tissues CREB-H appears to be specifically restricted to liver tissue, although lower levels have been detected within the small intestine by RT-PCR (12, 81, 120). CREB-H is a glycosylated protein of the ~76 kDa and, as with the other family members, is cleaved in an S1P- and S2P-dependent manner (12, 120), generating a 42-kDa nuclear form.

CREB-H binds CRE sites and to B-Box elements *in vitro* (81). The B-Box element was initially identified in the *Drosophila* ADH gene, and acts as an enhancer, specific to the fat body, equivalent to the mammalian liver. In additional studies of DNA-binding specificity, CREB-H was reported to bind to CRE, ATF6, and B-Box elements, with reduced binding to ERSE-1 and ERSE-II sequences (117). Consistent with CRE-

recognition, CREB-H activates transcription from the CRE-containing PEPCK promoter (117), although the precise physiologic role of this is unclear (120). CREB-H, consistent with its ability to bind to ATF6 sites with the motif TGACGTGG/A (111), efficiently activates transcription from an a ATF6 promoter–reporter construct (12, 120). Compared with the ATF6 UPRE binding site to which it binds well, CREB-H binds the ERSE site very poorly. Consistent with this, CREB-H does not appear to activate transcription from artificial promoters containing the ERSE element or from the classic BiP or GRP94 chaperone promoters (12, 120). These results indicated that CREB-H may not serve as a classic UPR trans-activator and that it has other roles in mediating stress responses.

In a recent study, CREB-H has been show to act in the systemic inflammatory response and potentially to function in integrating UPR and inflammatory responses (120). In this study, gene-chip analysis identified CREB-H as upregulating, among others, two genes; C-reactive protein (CRP) and serum amyloid P component (SAP). CRP and SAP are liver-specific genes, induced during the acute-phase response, in response to proinflammatory cytokines or bacterial LPS (78). Consistent with this, CREB-H mRNA levels were upregulated in a liver-derived cell line in response to treatment with the inflammatory cytokines, IL-6, IL-1β, or TNF-α (120). CREB-H was cleaved in an S1P- and S2P-dependent manner, by agents that typically induce ER stress, including tunicamycin, thapsigargin, and DTT. This was associated with induction of CRP and SAP expression, suggesting that ER stress impinges on and activates not only the UPR pathway but also (at least within the liver) the acute phase–response pathway (120). CREB-H was also observed to interact synergistically with ATF6, in activating transcription from CRP and SAP promoters, and this was attributed to heterodimeric interactions between ATF6 and CREB-H, as demonstrated by IP (120). Finally, the same authors demonstrated that CREB-H (and ATF6) bind to a conserved sequence found within the CRP and SAP promoters, with a motif of GACGTG, which is highly similar to the ATF6 (TGACGT) and CRE (TGACGTCA) binding elements. This study highlighted an additional important function in which ATF6 could act as a general stress factor that modulates the activity of the other tissue-specific membrane-bound transcription factors, by acting as dimerization partner, thereby furthering the spectrum of responses that these factors can mediate.

CREB-H therefore appears to have an important role in regulating the inflammation response. However, many questions remain on the mechanism involved and how it relates to previous results on ATF6, BiP involvement, and stress sensing. One possibility is that cell-type–specific differences affect the magnitude and range of cellular responses. The study (120) highlighted a fundamental difference in the acute inflammatory response *in vivo* compared with *in vitro* cell culture models. Cell-type–specific differences could also be involved in modulating the activity and response of another member of the RIP family, CREB4.

CREB4/CREB3L4

CREB4 (CREB3L4) was originally identified as part of a general sequencing initiative, with transcripts detected in mainly human prostate, but also in other tissues including brain, pancreas, skeletal muscle, small intestine, testis, leukocytes, and thymus (9). The same protein, known as AIBZip, was also identified in a screen for genes induced in androgen-treated prostate cancer cell lines (85). In contrast, the mouse homologue of CREB3L4, also termed ATCE1 or TISP40, was identified in a screen for factors involved in spermatogenesis, and shows a highly restricted testis-specific expression profile (1, 71, 85). Human CREB4 protein migrates at ~53 kDa, larger than expected, indicating that CREB4 is also glycosylated (71), whereas in mice, two isoforms (α and β) are predicted, differing by an N-terminal extension of ~50 aa (1, 71), with the shorter (α) form the most abundant in mouse testis (18, 103).

With regard to DNA binding, an initial report indicated that murine CREB4 recognized the NF-κB element of the IL-2 receptor (103), although this has not been confirmed in another study. Instead, murine CREB4 binding to the UPR element (-TGACGTGG-) was observed, and this was specific, in that the closely related CRE (-TGACGTGG-) was not recognized (71). Consistent with the binding studies, CREB4 activated expression from a target gene containing multiple UPR elements. Activation was not observed on a promoter containing the ERSE, nor did murine CREB4 induce ERSE-containing genes BiP or GRP94. CRE binding was, however, observed when CREB4α was present as a heterodimer with CREM, a key transcriptional regulator of spermatogenesis (72). It should also be noted that of the two distinct CREB4 isoforms, the β form is a potent transcriptional activator, in contrast to the α isoform (71), and therefore, dimerization could be a major mechanism regulating the ability of CREB4 to activate transcription in the murine model. Human CREB4 has been shown potently to activate expression from a target gene containing multiple UPR elements, with some but weaker activation observed on the native BiP promoter (104).

Limited information is available for the functional role of human CREB4, and potential extrapolation from studies of the murine homologue must be tempered by the observation of different patterns of tissue-specific expression. Studies on human prostate cells have found that, in response to androgen stimulation, CREB4 mRNA and protein levels are upregulated in a dose-dependent manner (85). In addition, CREB4 is expressed at lower levels in normal prostate cells and at higher levels in cancerous prostate, particularly epithelial prostate cells. CREB4 mRNA was also detected in breast cancer cell lines, suggesting that CREB4 may be expressed in hormone-sensitive cell types (85). It is therefore interesting to speculate that CREB4 responds to stresses that are induced as a result of hormone action, or that a cell-type–specific regulation exists for CREB4 activation.

In mice, CREB4 exhibits restricted testes-specific expression and appears within the germ cells during the haploid stage of spermatogenesis (1, 103), where it may modulate late spermatid gene expression. An intriguing hypothesis with regard to cleavage by S1P and S2P is that CREB4 may be stored in the sperm head, where it is delivered by fertilization to the zygote, and then function as a zygotic or early embryonic activator (23). However, studies on CREB4 knockout mice suggest a more direct but subtle role in spermiogenesis. CREB4 knockout mice show a normal phenotype in survival, behaviour, and gross morphology. They also exhibit no apparent defect in fertility, but

did have reduced levels of spermatozoa (1, 73). Reduction in levels of spermatozoa correlated with increased apoptosis in the germ cells (1) and increased activation of the apoptotic activator, Caspase-12 (73). Apoptosis induced by caspase 12 is a downstream terminal response to excess ER stress (74), and these results suggest a role of CREB4 in responding to ER-stress events arising during spermiogenesis, a developmental event in which substantial reorganization of the ER and Golgi occurs.

The details and mechanisms of CREB4 regulation and stress signalling also are limited. Unlike the other RIP family members whose location in the absence of stress is predominantly the ER, in transient expression of human CREB4, the protein exhibited significant constitutive localization to the Golgi, but with no nuclear staining or cleavage observed (85, 104). Based on the paradigm established from studies with ATF6, Golgi localization would be expected to result in cleavage, as a result of processing by S1P and S2P. CREB4 therefore appears to be resistant to S1P/S2P-mediated cleavage, under these conditions. Further analysis indicated that CREB4 could be cleaved by S1P, but only after deletion of a region of the C-terminal luminal domain, or by exchanging this region with that from Luman (104). The results suggested that the CREB4 C-terminal luminal domain acts as negative regulator of S1P-mediated cleavage. Because the S1P motif CREB4 (S)RxI is a weaker match to the refined consensus (S)RxL, more subtle regulation of S1P activity (104) may occur, or requirement for a different protease or tissue-specific splice variant.

BBF2H7/CREB3L2

BBF2H7/CREB3L2 is the least-characterized family member. CREB3L2 was identified by virtue of its involvement in a gene translocation in low-grade fibromyxoid sarcoma, resulting in fusion to the FUS gene on chromosome 16 to the CREB3L2 gene on chromosome 7 (105). RT-PCR analysis suggests that CREB3L2 is widely expressed in most tissues, although at very much reduced levels in heart, brain, and colon (105). More recent Northern blot analysis also demonstrated widespread tissue expression, but highest levels of expression were found in heart and placenta, not completely consistent with the earlier RT-PCR studies (82). CREB3L2 is predicted to encode a protein of 57 kDa encompassing the conserved bZIP-TM, S2P site, and S1P sites. Alignment analyses indicated that it is more closely related to OASIS than other family members, with significant short regions of homology between the two outside the conserved core domain.

Characterization of CREB3L2 has largely remained within the clinical setting, with the identification of the chromosomal translocation t(7;16)(q32–34;p11) in low-grade fibromyxoid sarcoma, a translocation specifically associated with this type of tumor (64, 83). The translocation results in the CREB3L2 bZIP domain and C-terminal domain being fused to the N-terminal FUS gene. Recent results indicate that CREB3L2 can activate expression from target promoters containing either UPRE, CRE, or B-Box elements (82). Activation of the native BiP promoter was also observed. Interestingly, the constructs encoding the chimeric FUS/ CREB3L2 proteins exhibited the most potent activation, even in the context of full-length proteins encompassing the transmembrane and luminal domains of CREB3L2. Localization studies indicated that CREB3L2 was located in cytosolic reticular structures likely to represent the ER (82). Recent results have shown that CREB3L2 is indeed localized to the ER and that ER stress induces its translocation to the Golgi and cleavage (46). CREB3L2 protein was virtually undetectable in the absence of stress and was induced in response to stress at the translational level. ER stress normally initially suppresses translation *via* the PERK/eIf2α pathway, with unusual proteins, such as ATF4, escaping suppression and being translated as a result of a specific gene organization in the untranslated region. However, the specific translational induction of CREB3L2 does not appear to be *via* the PERK pathway, suggesting interesting new avenues for the regulation and for the role of this factor. Furthermore, although CREB3L2 appears to protect cells from stress-induced cytotoxicity, it does not appear to induce the expression of endogenous BiP, suggesting alternative roles and target pathways in stress-induced signaling (46).

EVOLUTIONARY ASPECTS OF bZIP-TM PROTEINS

The mechanisms involved in signaling UPR become more complicated as we consider the components from yeast to higher metazoans. In yeast, the key pathway is signaled *via* IRE1 and HAC1, and although IRE1-independent stress responses seem to be present (47), no evidence exists for either a PERK-related or ATF6-related pathway. In *Caenorhabditis elegans*, a PERK-related protein appears (PEK) contains a kinase domain juxtaposed to a domain related to the IRE1 luminal domain. The presence of PEK represents the appearance of a signaling route to suppress translation in response to ER stress at the membrane by phosphorylation of translational initiation factors (3). *C. elegans* also contains two bZIP proteins with adjacent transmembrane domains, and although the degree of homology is less than that within the mammalian family, these proteins are most closely related to ATF6 and CREB-H. In mammals, the bZIP-TM family expands to six members: ATF6α, ATF6β, Luman, OASIS, CREB4, CREB-H, and BB2H7, with expression patterns ranging from broad to quite highly tissue specific.

Examination of the effect of knockdown of ATF6, CREB-H, and other components of the UPR pathway in *C. elegans* (102), together with analyses of the consequence of ATF6 knockdown in mammalian cells (48), has led to some revision of the functional relevance of these components in UPR and the potential involvement in other networks. In *C. elegans*, ATF6 deficiency had no significant effect on viability, phenotype, or the UPR response. From microarray studies, the expression of only six genes was directly affected by loss of ATF6, and these were not involved in protein folding, secretion, or ERAD (102). ATF6 was, however, essential in the absence of the IRE1/XBP pathway. Independent loss of ATF6 in *C. elegans*, although having few obvious phenotypic consequences, did, however, affect gene expression, resulting in the reduction of a number of genes within the class termed constitutive UPR

genes (cUPR) (102). These genes had a range of roles, and the results suggest the involvement of ATF6 in normal cell processes or development or both. In contrast to the dispensability of ATF6, CREB-H appears to be an essential gene in *C. elegans*, with siRNA knockdown resulting in embryonic lethality. CREB-H expression is itself induced during stress, in an IRE-1/XBP-1–dependent manner, but as yet, we have no information on the nature of the requirement for CREB-H in developmental or adaptive responses in *C. elegans*.

Studies in mammalian cells are consistent with the apparent lack of a requirement for ATF6 in mediating UPR in *C. elegans*. Depletion of ATF6α, whether alone or even in combination with ATF6β, did not significantly affect the UPR response, as assessed by gene profiling (48). Much of the analysis of the novel bZIP-TM family originates from the observations that ATF6 can regulate at least a subset of UPR genes. Not only does it remain an open question as to the extent of the ATF6-responsive genes and the relative importance of its optimal recognition site (the UPRE) *versus* the ERSE I and II, but also the conclusion from knockdown experiments is that ATF6 may play a redundant or auxiliary role. This notwithstanding, it has been reported that cells lacking the processing enzyme S2P are themselves unable to support a UPR (50). If ATF6 is redundant or plays an auxiliary role, this raises the question as to the nature of the requirement of S2P. It may, therefore, be that one of the other new members of the family, all S2P substrates, actually plays a more important role than ATF6. Clearly many questions remain on the relative contribution of ATF6 and these factors, not only in the general responses shared by all cells, but also in cell-type–specific stresses and the integration of these pathways.

Whereas several of the bZIP-TM members have been shown to respond to general ER stress, such as that induced by tunicamycin, other reports indicate selectivity in response, with, for example, Luman being cleaved and activated in response to thapsigargin, but not tunicamycin. Selectivity in stress sensing at the ER rather than broad-spectrum activation of each of these factors is more likely to reflect the physiologic situation. One example of this comes from recent studies on human CREB-H, whose cleavage is induced in response to proinflammatory signals, including those induced by IL-6 and dTNFα. (120). CREB-H activation appears to induce expression of a distinct class of genes including those for C-reactive protein (CRP) and serum amyloid P component (SAP), rather than typical UPR proteins. Moreover, the N-terminal CREB-H product is not capable of activating from either ERSE elements, nor does it activate expression from the native chaperone promoters, including BiP, calreticulin, and Erp72. The preference of CREB-H for UPR-type elements and its failure to activate chaperone gene expression is consistent with studies on Luman (15), but contrasts with reports (*e.g.*, on OASIS) (45). Finally, CREB-H appears to be able to form heterodimers with ATF6, an interaction that confers synergistic activation on the CRP and SAP promoters. Taking these considerations together, it may be not only that the main role of these bZIP factors is in signaling ER-associated events apart from the classic UPR, but also that even for ATF6 itself, the main role is integrating responses to multiple stimuli, rather than being the direct key activator of transcription of UPR genes.

MECHANISMS OF REGULATED CLEAVAGE, POTENTIAL ROLES FOR BZIP-TM PROTEINS AND FUTURE ISSUES

The identification of potential transmembrane bZIP factors in *C. elegans* raises a number of questions, not only on general physiologic role, but also on detailed mechanisms. The current general model for cleavage of the ER-localized bZIP-TM pro-

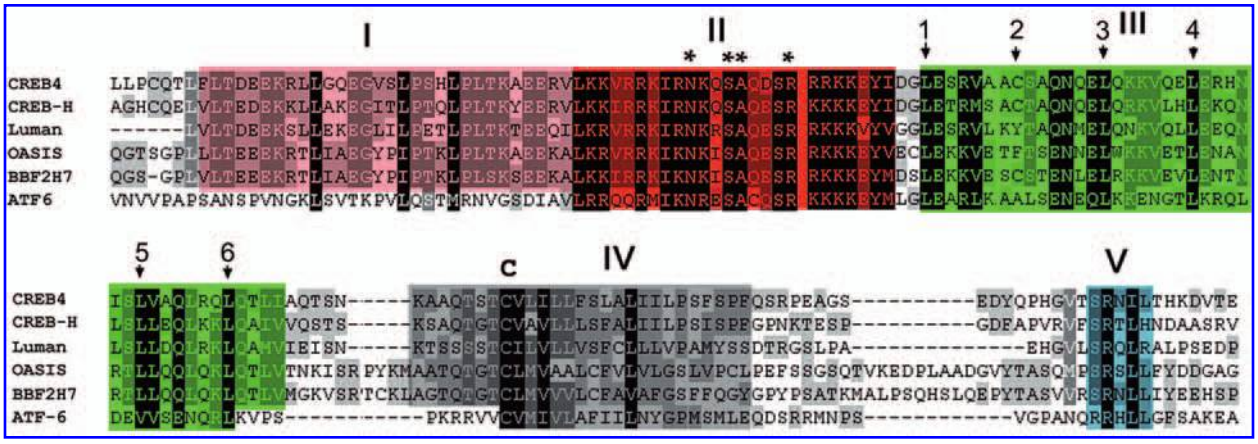

FIG. 3. Amino acid sequence alignments of the conserved domains. The subregions discussed in the text are colored as follows: region I (*pink*); region II, the basic domain (*red*); region III, the leucine zipper (*green*), region IV and the putative transmembrane domains (*grey*), and region V, the S1P site. The blocks indicate regions with identity or similarity in four of the five sequences, but shading has also been used to highlight features of interest. For example, whereas region I is not well conserved in ATF6, certain residues (*black background*) are conserved in all the proteins. Within region III, shading has been used to highlight similarities and differences. In region IV, a hydrophobic consensus motif contains a completely conserved cysteine, as discussed in the text. This is colored in *grey* with certain resides in *black*, for ease of reference. Region V, the S1P site, has certain refining features that indicate that it may represent a subset of S1P sites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

teins is based on the cleavage mechanism for SREBP. The general model is one of a two-stage process, in which the factors are first cleaved by S1P, a protease whose active form is restricted to the Golgi and with which they will not come into contact until forward transport is induced by stress. After S1P cleavage in the Golgi, the factors are subsequently cleaved by S2P, releasing the active cytosolic domains. Although *C. elegans* has now been shown to encode ATF6 and CREB-H orthologues, it nevertheless does not appear to encode a homologous S1P gene. Thus, *C. elegans* ATF6 and CREB-H proteins may be cleaved by an alternative protease before S2P cleavage, or the mode of regulation may be more direct, *via* S2P. Early work on ATF6 indicated a potential role for a protease other than S1P, because cleavage was still observed in a cell line that lacks S1P (30, 115). Similarly, recent studies have suggested that S1P cleavage is not essential before S2P cleavage for OASIS (70). Studies on ATF6 have indicated that the bulky luminal domain inhibits S2P-mediated cleavage, and that S1P cleavage is in effect to remove this inhibition (see Fig. 1). S2P cleavage remains, in this model, a Golgi-localized event otherwise ATF6 deletions lacking the luminal domain would be expected to be cleaved in the ER (100). Furthermore, recent studies on CREB4 have indicated that Golgi localization alone may not be sufficient to mediate S1P cleavage and that additional regulation of the cleavage events could take place in the Golgi (104). The involvement of an alternative luminal protease, additional pathways bypassing S1P cleavage, selectivity in S1P dependence/independence between different family members, and the regulation of cleavage activity in the Golgi are important questions remaining to be investigated. Further questions remain on the nature of homo- and heterodimerization of ATF6 and the related factors in the ER, the Golgi, and the nucleus. Although certain bZIP factors may not dimerize efficiently in solution, it may be expected that appropriate homodimers would form at the membrane and thus that the proteins may be regulated as dimers. Therefore, interactions involved in BiP binding, release, cleavage, and so on, must be considered in this context. For example, it may be expected that S1P would need to cleave at both sites to effect release of the dimer, a requirement likely also for S2P.

Furthermore, understanding the role of S1P in regulation and activation and this family of transcription factors may also shed light on the role of S1P itself. Defects in development in zebrafish cartilage have been observed in S1P and S2P knock-

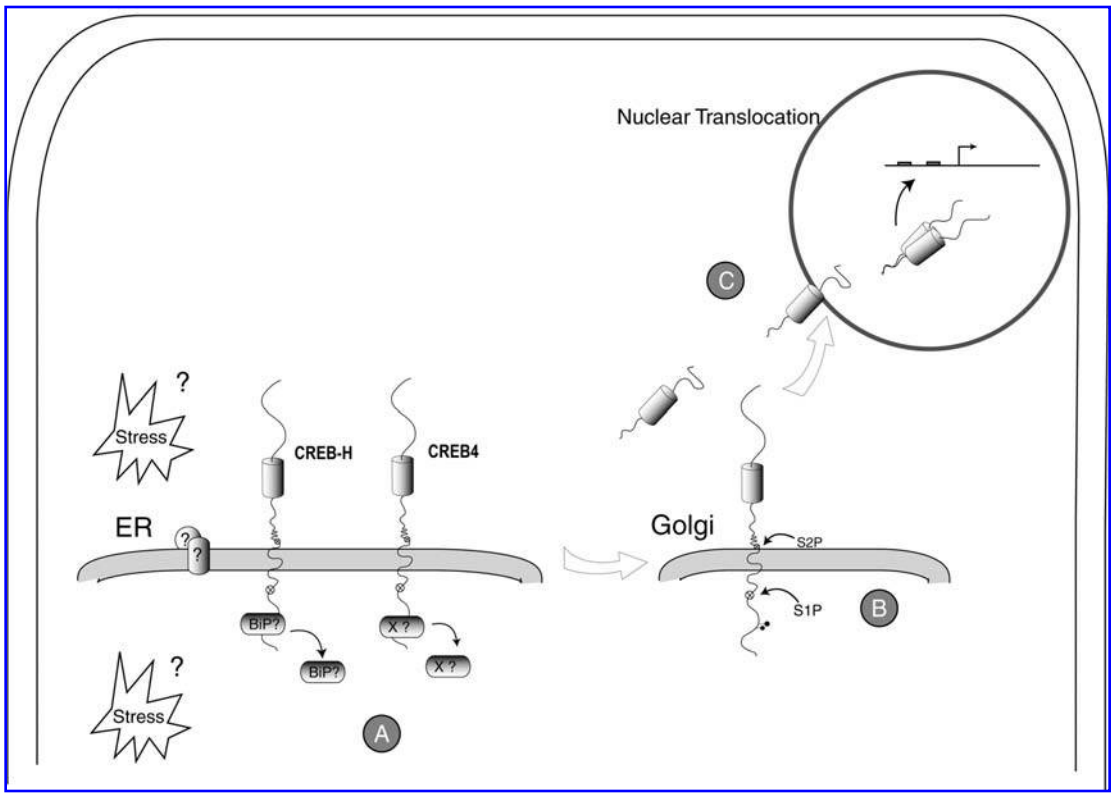

FIG. 4. Selectivity in stress recognition, regulation of transport, cleavage, and activation by bZIP-transmembrane family members. As discussed in the text, the expansion of the number of ATF6-related bZIP transmembrane factors, together with currently published results, indicate the possibility of differential stress recognition and associated adaptive responses. For example, as indicated (region A), the stresses involved in inducing transport and subsequent cleavage have not been identified and may not be the same; it is possible that certain factors could respond to cytoplasmic as well as or instead of luminal stress. In region B, cleavage by the Golgi-associated proteases may not be identical and could afford an additional point of regulatory control. In region C, nuclear transport, the possibility for heteroligomerization, and differential DNA recognition all contribute to expanding the possible mechanism of selectivity and integration with other transcriptional response pathways. These and other outstanding issues are discussed in the text.

downs, but this is not be readily attributed to defects in SREBP (97). Although other substrates and possibilities exist, this family of transcription factors could be involved in pathways of development and differentiation quite apart from adaptive/inflammatory responses. It is of note that in the *C. elegans* study described previously, the single gene that specifically required ATF6 for expression was *cht-1*, a chitinase orthologue (120). Chitinase-like proteins that are secreted in high levels have been specifically detected in human cartilage-producing cells (25, 32). Perhaps S1P and either ATF6 or other S1P-dependent members of this family of transcription factors are involved in regulating an evolutionarily conserved pathway.

ATF6 is generally considered to be one of the three arms mediating an integrated response to the accumulation of unfolded proteins, the other pathways being IRE1/Xbp and PERK (3, 10, 27, 38, 89). Although revised models have been proposed for IRE1 and PERK, for ATF6, direct interaction with BiP and stress-induced dissociation from BiP are regarded as the central mechanisms regulating forward transport and therefore cleavage (101). Considering the similarities between ATF6 and the broader bZIP family members, together with the reported activation for some of them of ER chaperones, including BiP, a key question concerns their mode of regulation. Are each of these factors first bound by BiP, and second, if so, is binding regulated by stress-induced dissociation? If these factors are not bound by BiP, in what way are they regulated, and is their role rather to sense some other aspect of ER stress with chaperones and similar genes not the relevant targets?

In this regard, the bZIP-TM members exhibit significant differences in tissue distribution, (accentuated in CREB4, in which the distribution in mice and humans seems radically different). Although, as for ATF6, certain members exhibit broad distribution, others such as CREB-H appear to be highly tissue specific. It may well be that, as suggested for CREB-H, the real role of these factors is in sensing different forms of ER stress, nutrient status, environmental perturbations, only operating in a particular cell type. A variant of this proposal for selectivity is that the ultimate signals may be similar, but the involvement of multiple factors allows a gain in control, whereby different members are sensitive to different levels of the same stresses. The operation of such controls affords the opportunity for integration of multiple pathways, for example, by virtue of differential heterodimerization of the cleaved products, creating different combinations and thus selective pathways of gene expression, catering to multiple integrated adaptive responses. Studies identifying differences in specific types of stress involved in eliciting transport and cleavage of different factors will aid our understanding of the family. Coupling such information with comparison of the target genes activated by different stresses, the mapping of response elements in different genes, and detailed analyses and comparison of the DNA-binding properties of the bZIP domains will help identify the role of individual and combinations of factors. Studies in yeast indicate that although fluctuation and stress mediated by diverse types of agents provoke universal responses, stress-specific responses are mediated by defined transcription factors (22). It is clear that in multicellular organisms, with specialized cells and tissues encountering radically different environments and organization, that stress-specific signaling is likely to be the rule rather than the exception.

CONCLUSIONS

SREBP and ATF6 are prototypical membrane-bound transcription factors that perform adaptive responses to different signals recognized by the ER, sterol levels, and the accumulation of unfolded proteins, respectively. Recent studies have highlighted new members of the class of membrane-anchored transcription factor proteins that are involved in stress signaling at the ER membrane. The ER–Golgi network is a diverse organelle, and further specializes in function in different cells and tissues of higher eukaryotes and will be subject to specific stresses or imbalances. For example, the liver expands the ER in response to various toxins, whereas the pancreas upregulates secretion in response to energy intake. As indicated schematically in Fig. 4, it is likely that current models for ER stress signaling are incomplete and that the expansion of the bZIP transmembrane family reflects selectivity in many aspects of these responses, including the type and duration of any particular stress, the cell type in which it occurs, and the integration with other aspects of cell-type–specific organization or additional intrinsic pathways, and the integration and communication between these pathways not only in a cell-type–specific manner, but between different tissues and organs. Many questions remain on the elucidation of the stress signals, the repertoire of components involved in regulating different aspects of the forward transport, cleavage, nuclear import, transcriptional activity and turnover of each of these factors, and dissection of the integration of the various outputs into broad coordinated responses. Such studies will lead to a more complete understanding of the molecular mechanisms involved in adaptive responses to diverse environmental stresses in different cell types, contributing to our understanding of physiologic and pathologic disease processes and providing novel avenues for the development of potential therapies.

ABBREVIATIONS

CRE, cyclic AMP response element; CRP, C-reactive protein; DTT, dithiothreitol; ER, endoplasmic reticulum; ERAD, endoplasmic-associated degradation; ERSE, endoplasmic reticulum response element; GRP, glucose-regulated protein; OASIS, old astrocyte specifically induced substance RIP, regulated intramembrane proteolysis; SAP, serum amyloid protein; SCAP, SPEBP cleavage-activating protein; SREBP, sterol regulatory element–binding protein; UPR, unfolded protein response.

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