The Luminal Domain of ATF6 Senses Endoplasmic Reticulum (ER) Stress and Causes Translocation of ATF6 from the ER to the Golgi

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ATF6 is an endoplasmic reticulum (ER) transmembrane transcription factor that is activated by the ER stress/unfolded protein response by cleavage of its N-terminal half from the membrane. We find that ER stress induces ATF6 to move from the ER to the Golgi, where it is cut in its luminal domain by site 1 protease. ATF6 contains a single transmembrane domain with 272 amino acids oriented in the lumen of the ER. We found that this luminal domain is required for the translocation of ATF6 to the Golgi and its subsequent cleavage, and we have mapped regions required for these properties. These results suggest that the conserved CD1 region is required for translocation, whereas the CD2 region is required for site 1 protease cleavage. We also find that ATF6's luminal domain is sufficient to sense ER stress and cause translocation to the Golgi when fused to LZIP, another ER transmembrane protein. These results show that ATF6 has a mechanism to sense ER stress and respond by translocation to the Golgi.

The endoplasmic reticulum (ER) is a critical cellular compartment responsible for the proper localization and folding of transmembrane and secreted proteins. Eukaryotic cells, from yeast to humans, have therefore developed mechanisms to ensure proper quality control and folding of proteins in this compartment (1). The cellular responses to unfolded proteins have been termed the ER stress or unfolded protein response. Nutrient deprivation and drugs that cause unfolded proteins in the ER can activate the ER stress response and allow the cell to survive the insult (1).

One part of the ER stress response is to activate new gene expression in the nucleus (2). Genes encoding ER chaperones, such as GRP78/BiP and GRP94, are transcriptionally activated such that their gene products are elevated in the ER and decrease the level of unfolded proteins (1). The analysis of promoters of several target genes has identified a consensus ER stress response element (ERSE) that is necessary and sufficient to mediate the activation of reporter genes by ER stress inducers (3, 4). The ERSE is bound constitutively by NF-Y, which is required for the inducible binding of ATF6 to the ERSE (5, 6). Both the ERSE and solo ATF6 binding sites can mediate ER stress induction of reporter genes (3, 4, 7). ATF6 is an unusual basic leucine zipper (bZIP) factor in that it contains a central transmembrane domain and is localized to ER membranes (8). It is activated by ER stress by cleavage from the membrane, freeing the N-terminal bZIP and transcriptional activation domains to move to the nucleus and activate transcription (3, 8). Dominant negative ATF6 constructs inhibited induction of target genes, suggesting that ATF6 is required for ER stress induction in vivo (5, 7). ATF6 is similar to the CREB-RP/G13 gene (recently renamed ATF6β) in the bZIP and transmembrane domains as well as in two regions in the lumen, termed CD1 and CD2 here (Fig. 1A) (8, 9). The role of CREB-RP is unclear; it appears to be activated similarly to ATF6, but its overexpression inhibited ER stress induction of the GRP78 promoter (3, 6, 10).

Sterol Response Element-binding Protein (SRE-BP) is a transcription factor that controls genes involved in maintaining cholesterol levels and is activated when cells are grown in low cholesterol (11). SRE-BP is similar to ATF6 in that it is also an ER transmembrane protein that is activated by cleavage from the membrane. SRE-BP is cleaved in a two-step process, first by site 1 protease (S1P) in the luminal loop of SRE-BP and second by site 2 protease (S2P) near the cytoplasmic end of the transmembrane domain (11). ATF6 is similarly regulated by S1P and S2P cleavage. An S1P-like site in ATF6's luminal domain and the S2P gene were required for ER stress-induced ATF6 cleavage (12). A cell line lacking S1P was partially defective for ATF6 activation, suggesting that it participates in ATF6 regulation but that there is another protease that can cleave the S1P site (12).

S1P is localized to the Golgi, and SRE-BP is activated to move from the ER to the Golgi in low cholesterol media (13, 14). This suggested that ER stress may induce ATF6 translocation to the Golgi, where it can be cleaved by S1P. We tested this hypothesis here and have mapped regions of ATF6 required for its activation. These results suggest that the luminal domain of ATF6 has an integral role in sensing ER stress leading to ATF6 cleavage in the Golgi and activation of target gene expression.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The HA-GALA-ATF6 expression series were constructed to contain SV40 or CMV promoters and an N-terminal HA epitope tag. They encode the N-terminal 147 residues of yeast GAL4 fused to the indicated regions of human ATF6. The S1P site mutation contains a change of amino acids 415 and 416 from RR to AA. The HA-ATF6 series was constructed in pCGN (15) and contains a CMV promoter, an N-terminal HA epitope tag, and the indicated regions of human ATF6. The M2-ATF6 plasmid was constructed in pDNA3.1 (Invitrogen) and encodes the human ATF6 with a FLAG tag at its N-terminus. The 3×FLAG-ATF6 plasmids were constructed in p3×FLAG-
CMV7.1 (Sigma) and encode the indicated regions of human ATF6 with three copies of the FLAG epitope tag at the N terminus. The GFP-ATF6 plasmids were constructed in pEGFP-C3 (CLONTECH) and encode green fluorescence protein N-terminal to the indicated region of ATF6. GFP-LZIP was constructed in pEGFP-C3 and encoded GFP in frame to the Nterminal of the LZIP (65-125) ORF. The GFP-LZIP-(1-280)-ATF6 (430-670) encodes GFP fused in frame to LZIP-(1-280) and ATF6-(430-670). The LZIP cDNA was kindly provided by Dr. Angus Wilson (New York University).

The Golgi marker DS1-GT was kindly provided by Dr. Karin Schwab (DKFZ, Heidelberg) and encodes red fluorescent protein fused to the transmembrane region of galactosyltransferase. S1P-KDEL and S1P-XX of Dulbecco’s modified Eagle medium with 10% newborn calf serum. NIH3T3 cells were stably expressing HA-tagged ATF6 were prepared by retroviral infection and selection with 1.5 μg/ml puromycin. The virus expressing ATF6 was generated by transfection of 293 cells with pBABE-puro-ATF6 and a packaging site-defective Moloney murine leukemia virus.

Dual Luciferase Assay—HeLa cells were set at 1 × 10⁶ cells/35-mm dishes 24 h prior to transfection. The cells were transfected by the calcium phosphate-DNA coprecipitation method as described (18). Each transfection mixture for ATF6, pBabe-puro-ATF6, was constructed with an HA epitope tag at its N terminus in pBabePuro (17).

Cell Culture—HeLa and NIH3T3 cells were grown in Dulbecco’s modified Eagle medium with 10% newborn calf serum. NIH3T3 cells stably expressing HA-tagged ATF6 were prepared by retroviral infection and selection with 1.5 μg/ml puromycin. The virus expressing ATF6 was generated by transfection of 293 cells with pBABE-puro-ATF6 and a packaging site-defective Moloney murine leukemia virus construct.

GFP and Immunofluorescence—For detection of GFP-ATF6, HeLa cells were grown on uncoated 22 × 22-mm number 1 coverslips (Fisher) in a 60-mm dish (Figs. 4, 5, and 7) at 2 × 10⁶ cells/dish. After 24 h, cells were transfected with 20 μg of GFP-ATF6 constructs alone or together with 4 μg of Golgi marker construct DS1-GT. 20 h later, cells were washed with PBS and grown in fresh Dulbecco’s modified Eagle’s medium with 10% newborn calf serum for 4 h. A slide coverslip chamber was prepared as described (19). Briefly, two strips of double adhesive tape (1.5 × 30 mm) were placed on both edges of the slide, and then the coverslip, on which HeLa cells were growing, was pressed onto the adhesive tape with the cells facing downward into the chamber. Rapid exchange of media was affected using a wick system as follows. A drop of Dulbecco’s modified Eagle’s medium plus 10% newborn calf serum (with 25 μM HEPES, pH 7.3), with or without 10 mM DTT, was placed at the end of one open edge of the slide coverslip chamber, and tapered strips of filter paper were placed in contact with the medium at the other open edge. The medium-exchanged chamber was sealed with low melting point wax around the four edges. The assembly was mounted on the microscope stage’s slide holder. The temperature was maintained at 37 °C as described (19). Fluorescence microscopy was carried out on an Optiphot-2 microscope at ×800 magnification with appropriate filters for fluorescence detection. Pictures were taken with a Hamamatsu digital camera with Metamorph software.

For immunofluorescence, HeLa cells were transfected onto coverslips in 35-mm dishes with 0.5 μg of FLAG-ATF6 and, where indicated, 0.5 μg of DS1-GT as a Golgi marker. 2 days after transfection, the cells were treated with or without 5 mM DTT for the indicated times, and the cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed with PBS for 5 min, and permeabilized and blocked with blocking buffer (0.1% Triton X-100 and 1% bovine serum albumin in phosphate-buffered saline-0.5 mM EDTA) for 1 h at room temperature. The cells were then washed twice with PBS at room temperature for 10 min each. The coverslips were mounted on microscope slides, and fluorescence microscopy was carried out on a Nikon Diaphot 300 at ×400 magnification with appropriate filters for fluorescence detection. Pictures were taken with a Hamamatsu digital camera with Adobe Photoshop software.

RESULTS

Regulated Activation and Cleavage of GALA-ATF6 through the S1P Site—ATF6 is activated and cleaved in response to inducers of the ER stress response such as tunicamycin (an inhibitor of glycosylation), thapsigargin (an inhibitor of an ER Ca²⁺-ATPase), and DTT (a strong reducing agent) (1). We have found that ER stress induction of ATF6 can be reproduced with a GALA-ATF6 fusion construct (7). We therefore mutagenized GALA-ATF6 to test for the requirement of different domains. We first tested the importance of the S1P site in the functional activation of ATF6. The S1P site in SRE-BP requires the sequence RXXL (20). We therefore mutated a similar sequence in ATF6, RRHLL (aa 415–419), to AAHLL. The GALA-ATF6 (aa 1–670) constructs were transfected into HeLa cells with a GALA site reporter gene. Wild type ATF6 mediated strong tunicamycin induction of the reporter gene, whereas mutation of the S1P site completely abolished induction (Fig. 1B). This regulation of GALA-ATF6 requires low level expression such that we have used the relatively weak SV40 promoter without an enhancer to express GALA-ATF6. Higher expression resulted in a low level of cleavage of ATF6, which is sufficient to activate the reporter gene in uninduced cells (data not shown).

However, it was difficult to detect GALA-ATF6 in immunoblots using the SV40 promoter. To compare expression and stability of the HA-tagged GALA-ATF6 variants, we expressed the same variants with a CMV promoter. In this way, we found that both GALA-ATF6 and GALA-ATF6/S1P⁻⁻ were expressed at similar levels (Fig. 1C).

We previously had difficulty detecting cleavage of ATF6 in response to inducers of ER stress such as tunicamycin (7). Possible reasons are that cleavage occurs at low levels and is not synchronous in response to tunicamycin. We used DTT as a stronger and more synchronous inducer of unfolded proteins in the ER (8, 21). We found that DTT was able to induce cleavage of GALA-ATF6 after transfection into HeLa cells and that the S1P site mutations blocked this cleavage (Fig. 1C).
Only a small amount of ATF6 cleavage was observed compared with full-length ATF6 after overexpression of GAL4-ATF6 in HeLa cells. To test whether lower levels of ATF6 would be more completely cleaved, we generated a stable cell line using a retroviral vector expressing ATF6 with an N-terminal HA epitope tag. In these cells, ATF6 was effectively cleaved in response to DTT with 80–90% of ATF6 cleaved after 1 h of treatment (Fig. 1D). Cleavage was also activated by another inducer of the ER stress response, thapsigargin, although the kinetics were slower (Fig. 1D).

Two cleavage bands of ATF6 were observed after DTT treatment (Fig. 1D). This is consistent with the two-step cleavage by S1P and then S2P (12). Cleavage by S1P would be predicted to result in a membrane-associated protein, while cleavage by both S1P and S2P frees the product to move to the nucleus. We tested whether the cleavage products conformed to this model by fractionation of the cell extracts. The upper cleavage product (labeled I) was in the membrane fraction, whereas the second product (N) was in both the membrane and nuclear extract fraction (Fig. 1E). It is not clear why the lower ATF6 product is in both the nuclear and membrane fractions. One possibility is that in some cases only a single monomer of the ATF6 homodimer is cleaved such that the cleaved monomer remains associated with the membrane through its dimerization with full-length or S1P-cleaved ATF6 (band I). It is also notable in Fig. 1D that there was not a clear precursor product relationship between the upper and lower ATF6 cleavage products. One explanation is that at least some of the S2P cleavage occurs quickly after S1P cleavage.

The C-terminal Domain of ATF6 Is Required for the Activation and Cleavage of ATF6—One possible model for ATF6 activation is that its luminal domain senses ER stress. A prediction of this model is that deletions of this domain that do not affect the S1P cleavage site will affect activation. Possible significant domains of ATF6 are the CD1 (aa 467–506) and CD2 (aa 550–640) regions that are conserved with CREB-RP (9).

We made a series of C-terminal deletions of GAL4-ATF6 and tested for their activation by tunicamycin (Fig. 2A). Deletion to aa 640 had no effect, but deletion into the CD2 region abolished activation. An internal deletion spanning the CD1 region (Δ430–550) also strongly reduced induction. The basal level of luciferase expression with each of these deletion constructs...
varied but was always in the low range (data not shown). Immunoblot analysis of the GAL4-ATF6 variants showed that they were expressed at similar levels (Fig. 2B and data not shown). We tested whether the C-terminal domain was required for ATF6 cleavage. Transfected HeLa cells were treated with or without DTT for 1 h. While a cleavage product of full-length ATF6 was detected in immunoblots, no cleavage product was observed for GAL4-ATF6-(1–600) or -(1–550) (Fig. 2B). These results further correlate activation and cleavage of ATF6 and suggest that the CD2 domain is required for S1P cleavage of ATF6.

Movement of ATF6 from the ER to the Golgi—S1P is localized in the Golgi, and SRE-BP must move from the ER to the Golgi in response to low cholesterol in order to be cleaved (13, 14). The role of S1P in ATF6 cleavage therefore suggests that ATF6 also moves out of the ER in response to ER stress. We tested this prediction with a FLAG-tagged ATF6 construct that was transfected into HeLa cells. We detected FLAG-ATF6 by immunofluorescence in the perinuclear and cytoplasmic regions in a manner consistent with ER localization (Fig. 3A). DTT treatment for 30 min caused the movement of FLAG-ATF6 to a perinuclear spot that is similar to Golgi staining. To test this, we coexpressed GFP-ATF6 with a Golgi marker, galactosyltransferase, fused to red fluorescent protein (DS1-GT). There was a strong overlap of the signals as shown by the merged images in Fig. 3B, suggesting that FLAG-ATF6 moved from the ER to the Golgi in response to DTT. At 60 min after DTT treatment of transfected cells, we found that FLAG-ATF6 had moved to the nucleus as expected for cleavage of ATF6 by S1P and S2P (Fig. 3A). The localization to the nucleus was confirmed by colocalization with a nuclear red fluorescent protein marker (data not shown).

The transient localization to the Golgi should be prolonged if S1P cleavage is blocked. We tested this by using FLAG-tagged ATF6 with mutations at the S1P site. This form of ATF6 moved to the Golgi similar to wild type ATF6 but was retained there even after 60 min of DTT treatment (Fig. 3C).

To provide further evidence that ATF6 moves to the Golgi, we used brefeldin A, which causes the relocation of Golgi proteins into the ER (22–24). For this purpose, we used a GFP-ATF6 construct with the S1P site mutated. The GFP fusion was used for ease of detection. Although it requires a higher level of expression to observe, we similarly found that GFP-ATF6 translocated to a perinuclear focus that colocalized with the Golgi-targeted DS1-GT (data not shown). The S1P site mutation was used, since, as with FLAG-ATF6, this caused a longer retention of GFP-ATF6 in a Golgi-like staining pattern after DTT treatment (data not shown). HeLa cells transfected with GFP-ATF6(S1P*) were treated with DTT for 1 h and then with or without brefeldin A (Fig. 4). DTT caused GFP-ATF6(S1P*) to move to the Golgi-like pattern. Brefeldin A then caused the pattern to disperse and return to a more ER-like pattern. This is consistent with a Golgi localization for ATF6 in DTT-treated cells.

Another method to test for Golgi localization is to probe for a
change in glycosylation with endoglycosidase H. N-Linked oligosaccharides in the ER are sensitive to endoglycosidase H cleavage. They become resistant to endoglycosidase H digestion in the Golgi after they are modified by α-mannosidase II, which is localized in the medial and/or trans-Golgi compartment in most cell types (25, 26). We did not detect clear resistance to endoglycosidase H digestion after DTT treatment (data not shown). While this does not show that ATF6 moves to the Golgi, it is consistent with movement to the cis-Golgi or an early compartment that does not contain α-mannosidase II.

We next tested whether the luminal domain of ATF6 was sufficient to cause translocation from the ER to the Golgi by fusing it to another ER transmembrane transcription factor. LZIP is a bZIP transcription factor that is localized to the ER and has a single transmembrane domain like ATF6 (27, 28). We found that GFP-LZIP gave an ER pattern as expected and did not translocate to the Golgi (data not shown). We also found that GAL4-LZIP was not activated by tunicamycin, suggesting that it is not an ER stress-responsive factor (data not shown). We fused the luminal domain of ATF6 to GFP-LZIP (aa 1-280), which contains LZIP’s cytoplasmic and transmembrane domains and 31 amino acids of its luminal domain. GFP-LZIP-(1-280) localized to the ER and was not induced to move by DTT (Fig. 5). However, fusion of ATF6’s luminal domain (aa 430-670) to GFP-LZIP-(1-280) caused it to move to the Golgi in response to DTT (Fig. 5). This demonstrates that the luminal domain of ATF6 is sufficient to sense ER stress and cause its translocation to the Golgi in response to DTT treatment. The efficiency of translocation of the fusion protein was not as high as with FLAG-tagged ATF6 (compare Figs. 3 and 5); however, this is at least partially due to the higher level of expression of the chimera, since GFP-ATF6 also did not translocate completely (Fig. 7).

**ER-localized S1P Causes Cleavage of ATF6**—The model in which ATF6 moves from the ER to the Golgi in order to be cleaved by S1P suggests that expressing S1P in the ER should cause ATF6 cleavage. We tested this prediction by expressing ATF6 with S1P that was tagged with the ER localization signal KDEL or, as a control, with a nonfunctional signal, KDAS (13). The fusion of KDEL onto S1P was previously shown to target it to the ER (13). S1P-KDEL, but not S1P-KDAS, caused cleavage of ATF6 at a position similar to the intermediate band (I) of DTT-induced cleavage of ATF6 (Fig. 6). There was a slight migration difference between these bands, suggesting that DTT and/or translocation of ATF6 to the Golgi may cause a change in post-translational modifications. There was no cleavage of the S1P site mutant ATF6, showing that S1P-induced cleavage requires this site (Fig. 6). These results show that S1P is capable of causing ATF6 cleavage if they are in the same cellular compartment.

**The CD1 Region Is Required for Translocation of ATF6 to the Golgi**—We mapped the region of ATF6 required for its translocation from the ER to the Golgi to determine whether a region is required for its retention in the ER or whether a positively acting sequence is required for its translocation. C-terminal deletions of GFP-ATF6 constructs revealed that the region between aa 468 and 500, containing the conserved CD1 domain, is required for movement to the Golgi (Fig. 7). GFP-ATF6-(1-467) was retained in the ER before and after DTT treatment, whereas GFP-ATF6-(1-500) translocated to the Golgi after DTT treatment. GFP-ATF6-(1-430) was localized in the ER, suggesting that the cytoplasmic domain along with the region spanning ATF6’s transmembrane domain is sufficient for localization to the ER. Deletions in the cytoplasmic domain suggest that the bZIP domain is required for ER localization, although this domain could be substituted by other bZIP domains.

To further establish that the CD1 region of ATF6 is required for its translocation, we made internal deletions in GFP-ATF6. We found that a variant with a deletion of the CD1 region (Δ468-500) did not translocate to the Golgi, whereas all of the other deletion mutants were able to translocate (Fig. 7).

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2 J. Shen and R. Prywes, unpublished results.
Correlation of the Requirement of ATF6 Sequences for Golgi Translocation and Functional Activation—We made similar mutations in GAL4-ATF6 as for the translocation assays in order to correlate activation of ATF6 by ER stress with translocation to the Golgi. The C-terminal 70 amino acids, which contain part of the CD2 domain, were required for activation of GAL4-ATF6 and S1P cleavage (Fig. 2) but not for translocation to the Golgi (Fig. 7). We also found that an internal deletion of part of the CD2 domain, Δ551–600, abolished induction of GAL4-ATF6 (Fig. 8A). Together, these results show that the CD2 domain is required for S1P cleavage and ATF6 activation but not translocation to the Golgi.

Internal deletions were used to determine whether other domains were required for activation. Deletion of CD1 (Δ468–500) caused a loss of translocation of ATF6 (Fig. 7) and also abolished tunicamycin activation of GAL4-ATF6 (Fig. 8A). This suggests that the translocation of ATF6 to the Golgi is required for activation of ATF6. We further found that this deletion of the CD1 region abolished cleavage as expected (Fig. 8B). Deletion of aa 431–466 had no effect on translocation to the Golgi or activation of GAL4-ATF6 (Figs. 7 and 8A).

Internal deletion of the region between CD1 and CD2, amino acids 501–550, gave a surprising result. This region was not required for ER localization or inducible translocation to the Golgi (Fig. 7). We found, however, that GAL4-ATF6-Δ501–550 was constitutively active, although this could be further increased by ER stress (Fig. 8A). This result suggests that this region of ATF6 negatively regulates its activation in uninduced cells. Since the deletion of aa 501–550 did not affect ER localization of GAL4-ATF6, it is surprising that it is activated without moving to the Golgi. We tested for cleavage of GAL4-ATF6-Δ501–550 and found that there was a low level of cleavage in uninduced cells (Fig. 8B). One explanation for these results is that a small amount of GAL4-ATF6-Δ501–550 can move to the Golgi in uninduced cells and that this amount was not distinguished from the general ER localization of GAL4-ATF6-Δ501–550 (Fig. 7). This would suggest that this region provides a secondary mechanism to block a low level of movement to the Golgi. However, the main mechanism to control ATF6 localization does not appear to require this region, since GFP-ATF6-1–500 and -Δ501–550 were inducibly translocated to the Golgi (Fig. 7).

The overexpression of GFP-ATF6 may mask the leakiness of low levels of ATF6-Δ501–550 to the Golgi. This is consistent with the sensitivity of our experiments to expression levels of ATF6. High level expression of ATF6 from a CMV promoter resulted in constitutive activation of GAL4-ATF6 and low levels of cleavage (data not shown). Lower levels of expression of GAL4-ATF6 from an SV40 promoter (without enhancer) produced GAL4-ATF6 that was strongly induced by tunicamycin as seen in Fig. 1. This sensitivity to ATF6 expression levels was previously seen where overexpression of ATF6 led to cleavage (8) and where low level expression from a thymidine kinase promoter was required to observe ER stress-induced cleavage (12). Together, these results suggest that only low levels of cleaved ATF6 are required for target gene activation.

**DISCUSSION**

ATF6 is activated during the ER stress response by cleavage resulting in the release of its cytoplasmic bZIP domain from the membrane and its translocation to the nucleus (8). This cleavage occurs in a two-step process by S1P and S2P proteases (12). We have found that ER stress induces ATF6 to move from the

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**Fig. 7.** The CD1 domain is required for translocation of ATF6 to the Golgi. HeLa cells were transfected with GFP-ATF6 constructs containing the indicated residues (1–670 for wild type (WT)) or the indicated internal deletions in full-length ATF6. The cells were treated with or without 10 mM DTT for 1 h. Typical images of the GFP fluorescence are shown.

**Fig. 8.** Functional activation of ATF6 deletion mutants. A, HeLa cells were transfected with SV40-GAL4-ATF6 constructs as indicated, treated with or without tunicamycin, and assayed for reporter gene activation as described in the legend to Fig. 1B. A diagram of the GAL4-ATF6 variants is shown. B, whole cell lysates of HeLa cells transfected with FLAG-tagged-ATF6 variants and treated with or without 10 mM DTT for 1 h were immunoblotted with anti-FLAG serum. The positions of full-length ATF6 (*), intermediate (I), and nuclear (N) fragments are shown as in Fig. 1.
ER to the Golgi, where S1P is located. Expression of S1P in the ER also resulted in ATF6 cleavage. Two lines of evidence further suggest that the luminal domain of ATF6 senses ER stress rather than acting as a passive substrate to proteases that are activated. First, deletion of various regions of the luminal domain abolished ER stress induction of GAL4-ATF6 constructs, translocation to the Golgi, and cleavage. Second, the luminal domain was necessary and sufficient for translocation of ATF6 from the ER to the Golgi. Together, these results suggest a model summarized in Fig. 9. ER stress induces a change in ATF6 or other factors that cause it to move to the Golgi. This requires the conserved CD1 region of ATF6. In the Golgi, ATF6 is first cleaved by S1P in the luminal domain near aa 418. This cleavage requires the CD2 region. ATF6 is then cleaved by S2P in the transmembrane domain or near the junction of the cytoplasmic and transmembrane domains. This allows the cytoplasmic bZIP domain to move to the nucleus, where it can bind ERSE target elements with the transcription factor NF-Y and activate expression of genes such as GRP78.

ER stress-induced translocation of ATF6 to the Golgi appears to be a critical step for its activation. We found that the CD1 region of ATF6 (aa 468–500) is required for its translocation and that this region is also required for the functional activation of GAL4-ATF6. Since deletion mutants of ATF6 that lack the CD1 region were retained in the ER, our results suggest that the cytoplasmic and transmembrane regions of ATF6 cause retention in the ER and that the CD1 region provides a positive signal for translocation. Deletion of other regions of ATF6 did not affect inducible translocation to the Golgi, suggesting that the CD1 region can both sense ER stress and mediate translocation of ATF6. The luminal domain of ATF6 was also sufficient to sense ER stress and cause translocation to the Golgi, as shown by the inducible translocation of an LZIP-ATF6 chimera. Our preliminary results suggest that the CD1 region alone along with some flanking sequence is sufficient for inducible translocation when fused to LZIP. Further work is required to more precisely determine the elements within this region necessary and sufficient for translocation to the Golgi. It is unclear how the CD1 region senses ER stress and causes movement to the Golgi. SRE-BP has provided a good model for how ATF6 is regulated. SRE-BP is an ER transmembrane protein that translocates to the Golgi in response to low cholesterol, where it is cleaved by S1P and then S2P (11). The translocation of SRE-BP requires a cofactor, SCAP, which senses low cholesterol and escorts SRE-BP to the Golgi (29). Whereas SCAP is not required for ATF6 translocation (12), there may be a similar “molecular escort” that binds ATF6.

Unfolded ER proteins are retained in the ER by their interaction with ER chaperones (1, 30). This type of retention mechanism is not supported for ATF6, since deletion of the CD1 region caused constitutive retention in the ER rather than allowing it to progress to the Golgi. IRE1 is also an ER transmembrane protein that is activated by ER stress (1). Activation of IRE1 appears to be inhibited by the binding of the ER chaperone GRP78, which is released when the levels of unfolded proteins rise (21). It is possible that an ER chaperone may bind to the CD1 region of ATF6 and cause its activation in response to unfolded proteins. In contrast to IRE1, this would have to be a positive signal, since the CD1 domain is required for translocation to the Golgi. Another possible model for ATF6 regulation is that the CD1 region is modified in response to ER stress and that this allows it to be recognized by the cellular machinery to translocate to the Golgi.

We previously found that overexpression of IRE1 activated ATF6 and that this activation was dependent upon IRE1’s kinase and nuclease activities (7). These results suggested that IRE1 is upstream of ATF6 in an ER stress signaling pathway. However, recent results with IRE1α and -β null cells have shown that these genes are not required for ER stress induction of GRP78 expression (31, 32). We have also found that IRE1 activation of GAL4-ATF6 did not require the S1P site or the C-terminal CD2 region of ATF6 (33). This contrasts with the requirement of the S1P site and CD2 for tunicamycin induction (Figs. 1 and 2) and further suggests that IRE1 is not upstream of ATF6 in the ER stress response pathway. The significance of IRE1 activation of ATF6 is unclear at this point, but it may be part of a separate pathway for activation of ATF6 target genes.

After ATF6 translocates to the Golgi, it is cleaved by S1P and then S2P. S1P requires the CD2 domain of ATF6 in addition to its cleavage site, suggesting that the cleavage reaction is more complicated. It will be important to reproduce this reaction in vitro to understand whether cofactors are required and how ATF6 is specifically recognized by S1P. The cleavage of ATF6 by ER-localized S1P (S1P-KDEL) resulted in the translocation of ATF6 (Fig. 5). This suggests that it is not subsequently cleaved by S2P in the ER. This is probably due to the localization of S2P in the Golgi, and we have observed such a localization of epitope-tagged S2P by immunofluorescence (2). In general, cleavage by S2P is poorly understood. S2P is a multipass transmembrane protein that cuts SRE-BP in the transmembrane domain (33). This is similar to γ-secretase and presenilin, which cleave amyloid plaque precursor in the transmembrane domain. Presenilin is similar to S2P in its topology in the membrane and requires a cofactor, nicastrin, to cleave amyloid plaque precursor (34). S2P may similarly require a cofactor for ATF6 cleavage. It also remains to be deter-

\[3\] X. Chen, J. Shen, and R. Prywes, unpublished results.)

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**Fig. 9. Model of ATF6 activation by ER stress.** See “Discussion” for details.
minded why S2P cleavage requires prior cleavage of SRE-BP and ATF6 at the S1P site. One proposal is that SRE-BP changes its structure within the membrane after S1P cleavage (35), but it is also possible that S2P or a cofactor recognizes the S1P-cleaved end. Future experiments need to be directed to understanding how these interesting proteases recognize and cleave their substrates.

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