Dependence of Site-2 Protease Cleavage of ATF6 on Prior Site-1 Protease Digestion Is Determined by the Size of the Luminal Domain of ATF6*

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ATF6 is an endoplasmic reticulum (ER) membrane-anchored transcription factor activated by regulated intramembrane proteolysis in the ER stress response. The release of the cytosolic transcription factor domain of ATF6 requires the sequential processing by the Golgi site-1 and site-2 proteases (S1P and S2P). It has been unclear why S2P proteolysis relies on previous site-1 cleavage. One possibility is that S2P localizes to a different cellular compartment than S1P; however, here we show that S2P localizes to the same compartment as S1P, the cis-medial-Golgi. In addition, we have re-localized S1P and S2P to the ER with brefeldin A and find that the sequential cleavage of ATF6 is reconstituted in the ER. The mapping of the region of ATF6 required for sequential S1P and S2P cleavage showed that short luminal domains resulted in S1P-independent S2P cleavage. The addition of artificial domains onto these short ATF6 luminal domains restored the S1P dependence of S2P cleavage, suggesting that it is the size rather than specific sequences in the luminal domain that determines the S1P dependence of S2P cleavage. These results suggest that the bulky ATF6 luminal domain blocks S2P cleavage and that the role of S1P is to reduce the size of the luminal domain to prepare ATF6 to be an optimal S2P substrate.

Regulated intramembrane proteolysis (RIP)†1 is a mechanism for signal transduction across membranes that involves the proteolytic processing of membrane-anchored transcription regulators. When the transmembrane signaling factors are activated, they can be cleaved by intramembrane-cleaving proteases (I-CLiPs) within the plane of the membrane. This cleavage liberates cytosolic fragments that enter the nucleus to regulate gene transcription (1–3). RIP represents a direct signaling pathway from the membrane to the nucleus and marks cellular events that enter the nucleus to function as a transcriptional activator. S2P is a Zn2+ metallopeptase categorized to the rapamycin-sensitive or cascades of phosphorylation.

One of the RIP-regulated factors is the endoplasmic reticulum (ER) membrane-associated basic leucine zipper transcription factor ATF6 (4). ATF6 controls the expression of genes involved in ER protein folding and trafficking. It is synthesized as an ER membrane-tethered precursor and activated by intramembrane proteolysis in a signaling pathway known as the ER stress response or unfolded protein response (5, 6). The ER is the site for the synthesis and folding of proteins destined to enter the secretory pathway, and perturbations in the ER environment can lead to the accumulation of misfolded proteins in the ER lumen (so-called ER stress) (7–9). ER stress induces a highly coordinated adaptive response including up-regulation of a number of genes involved in protein folding, trafficking, and degradation. ATF6 activation plays an essential role in the transcriptional induction of the ER stress-inducible genes (4, 10).

A general feature of RIP is that the proteolysis is tightly regulated and that this regulatory step usually includes a juxtamembrane cleavage preceding the intramembrane proteolysis. Examples of this include the cleavage of notch and amyloid precursor protein by presenilin, sterol regulatory element-binding protein (SREBP) by site-2 protease, and signal peptides by signal peptide protease (1). In each of these cases, a primary cleavage reaction removes much of the luminal domain and allows the subsequent cleavage by an intramembrane protease. After the first cleavage trims off the extracellular domain, the second cleavage within the lipid bilayer appears to be automatic, suggesting that the first cleavage reaction is the critical regulatory step (2). Tight regulation of ATF6 is conferred by its controlled translocation from the ER to the Golgi where active site-1 protease (S1P) resides. This trafficking is controlled by the ER chaperone Bip/GRP78 that binds to ATF6 and dissociates in response to ER stress (6, 11). The activation of ATF6 in the Golgi involves a two-step cleavage process typical to many RIP events. In the first step, ATF6 is cut within its luminal domain by S1P, a membrane-bound serine protease of the subtilisin family with its active site in the Golgi lumen. This step separates ATF6 into two halves. The N-terminal membrane-associated fragment retains a short luminal sequence (~20 residues) and is further cleaved by site-2 protease (S2P) within its transmembrane region, yielding a free cytosolic fragment that enters the nucleus to function as a transcriptional activator. S2P is a Zn2+ metallopeptase categorized to the rapidly expanding family of I-CLiPs that all have their catalytic sites embedded within the lipid bilayer (12).

The processing of ATF6 by S2P strictly requires prior cleavage by S1P. In S1P mutant cell lines or where S1P activity was blocked, S2P failed to process ATF6 into its active form (4, 6, 11, 13). In addition, ATF6 bearing mutations in its S1P cleavage site was not processed by S2P (4, 11). There are several
possible models to account for this dependence that is also seen with other RIP-regulated factors (1). First, S2P may be localized in the cell differently than S1P or ATF6 and cleavage by S1P may be required for ATF6 to move to the location of S2P. Second, specific sequences in ATF6 may block S2P cleavage until they are removed by S1P digestion. These sequences could inhibit ATF6 by specifically interacting with S2P or by sterically blocking its access to the cleavage site. In the case of a necrotic pro-cleaving the size of its luminal domain seems to block its intramembrane cleavage by presenilin, suggesting that inhibition may be by steric hindrance (14). However, in the case of the bacterial substrate RseA and the intramembrane protease YaeL, specific glutamine-rich sequences in the luminal domain of RseA were required to block YaeL digestion (15). A third model is that cleavage by S1P could create a substrate site for S2P binding and cleavage. In this study, we sought to distinguish these models.

EXPERIMENTAL PROCEDURES

Materials and Constructs—Brefeldin A (BFA), 4-2-aminoethylbenzenesulfonyl fluoride (AEBSF), anti-FLAG M2 antibody, anti-mouse IgG, fluorescein isothiocyanate-conjugated anti-mouse IgG, and horseradish peroxidase-conjugated goat anti-mouse sera were obtained from Sigma. ECL plus reagents were from Amersham Biosciences. Mouse monoclonal anti-HA antibody was from Covance.

ATF6 and its derivatives were cloned into the p3x-FLAG-cytomegalovirus 7.1 or 14 vectors (Sigma) to generate constructs encoding fusion proteins with three tandem copies of the FLAG epitope at their N termini (vector 7.1) or C termini (vector 14). cDNAs for P-GFP, SREBP2, and immunoglobulin heavy chain were kindly provided by Drs. Randal Kaufman, Michael Brown and Joseph Goldstein, and Linda Hendershot, respectively. The Golgi marker DSI-GT was kindly provided by Dr. Karin Schwab (German Cancer Research Center, DKFZ, Heidelberg, Germany) and encodes red fluorescent protein fused to the transmembrane region of galactosyl transferase.

Cell Culture, Transfection, and Immunoblotting—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum at 37 °C in a 5% CO2 incubator. HeLa cells were transiently transfected using the standard calcium phosphate DNA precipitation method as previously described (11). Cells were rinsed once in ice-cold phosphate buffered saline (PBS) 20–24 h after transfection and then lysed in SDS loading buffer (2% SDS, 60 mm Tris-HCl, pH 6.8, 10% glycerol, 0.001% bromphenol blue, and 0.33% β-mercaptoethanol). Proteins were separated on SDS-PAGE and probed with anti-FLAG M2 antibodies as previously described (6).

Indirect Immunofluorescence and GFP Microscopy—HeLa cells seeded and transfected on coverslips were fixed for 10 min in 4% paraformaldehyde in PBS and permeabilized for 10 min in 0.1% Triton X-100 in PBS. Cells were blocked overnight in PBS with 1% bovine serum albumin and subsequently incubated with primary and secondary antibodies diluted in PBS at room temperature. Mouse monoclonal anti-HA (1:200) was used as the primary antibody, and fluorescein isothiocyanate-conjugated anti-mouse IgG (1:50) was the secondary antibody. The coverslips were mounted on microscope slides and observed under the Nikon Diaphot 300 microscope at 4× magnification with appropriate filters for fluorescence detection. To visualize GFP fusion proteins, living cells maintained in tissue culture medium were observed under the same fluorescence microscope.

RESULTS

Localization of S2P—One model for sequential cleavage of ATF6 is that S1P and S2P reside in different membrane compartments and that, after S1P cleavage, the ATF6 intermediate fragment can move to the S2P compartment. We sought to test this model by probing where S2P can cleave ATF6. The first clue of S2P distribution came from our studies on an ATF6 variant (amino acids 1–430), which has a short luminal domain (~30 aa), similar to the intermediate form of ATF6 (ATF6-(1–418)) that results from S1P proteolysis (Fig. 1A). When tran-
cannot act on intact full-length ATF6 in the absence of S1P activity (Fig. 3A). Therefore, our reconstitution of the two-step ATF6 processing in the ER accurately recapitulated the dependence of S2P proteolysis on previous site-1 cleavage.

![S2P Digestion of ATF6](image)

**FIG. 1.** The ATF6 intermediate form is not spontaneously processed to the mature form in the ER. A, diagram of constructs encoding ATF6, SREBP2, and their derivatives. ATF6 and SREBP2 proteins are aligned to show the similarity of the two proteins. The transcriptional activation (TAD), DNA binding (DBD), and transmembrane (TM) domains are indicated. The approximate site-1 and site-2 cleavage sites are also marked. B, proteolytic processing of wild type (wt) and variant ATF6-(1–430). HeLa cells were transiently transfected with the indicated plasmids, and the 3×FLAG-tagged proteins were detected by immunoblotting of whole cell lysates. Cells were either untreated or treated with 5 mM DTT for the indicated times (min) or 5 μg/ml BFA for 1 h. The mature (M, S2P cleavage product) and intermediate (I, S1P cleavage product) forms of ATF6 are indicated. C, cellular localization of ATF6 and SREBP2 variants. The indicated GFP-ATF6 and GFP-SREBP2 fusion proteins were transiently expressed in HeLa cells. GFP-SREBP2-(21–530)S2P−, a variant with mutations in Asn-Pro residues in its transmembrane domain that block S2P digestion, was transfected with DS1-GT. DS1-GT encodes red fluorescent protein fused to the transmembrane region of galactosyltransferase and was used as a Golgi marker protein. The merged images of the GFP and red fluorescent protein signals are shown. Cells transfected with GFP-SREBP2(21–530)S2P− were also treated with 5 μg/ml BFA where indicated.

**FIG. 2.** Localization of S2P to the Golgi apparatus. HeLa cells were transfected with HA-tagged S2P and DS1-GT as a Golgi marker (top). Cells expressing HA-S2P were either untreated or treated with BFA for 1 h (bottom). The HA-S2P protein was visualized by indirect immunofluorescence with anti-HA antibodies.

**FIG. 3.** S1P-independent cleavage of ATF6. HeLa cells transiently expressing wild-type ATF6 (A) or the ATF6-(1–430) mutant (B) were either untreated or treated with 5 mM DTT for 30 min or 5 μg/ml BFA for 1 h before lysis for immunoblotting with anti-FLAG antibodies. Where indicated, AEBSF (500 μM) was added for 1 h to ablate S1P activity before applying DTT or BFA. Intermediate (I, S1P-cleaved) and mature (M, S2P-cleaved) forms of ATF6 are indicated.

Whereas wild-type ATF6 was not cleaved by S2P when S1P activity was blocked, we found that the ATF6 variant (ATF6-(1–430)) could be processed by S2P in cells treated with AEBSF (Fig. 3B). Whereas ATF6-(1–430) is similar to the S1P-cleaved intermediate ATF6 form (amino acids 1–418), it is not identi-
cal, suggesting that the precise formation of this form is not necessary to create an S2P recognition site. Rather, the cleavage of ATF6-(1\text{–}430), independent of S1P digestion, suggests that the luminal domain of ATF6 normally blocks S2P cleavage.

We next sought to determine the mechanism of the inhibitory effect by mapping regions of ATF6 luminal domain that block S2P proteolysis. As noted in the Introduction, cleavage of the RIP substrates Notch and RseA by the intramembrane proteases is also blocked by the luminal domains of the substrates but by markedly different mechanisms. For Notch, the general size of the luminal domain appears to be critical while specific glutamine-rich sequences in RseA blocked its site-2 digestion by YaeL (14, 15). We constructed a series of C-terminal deletion mutants of ATF6 and examined whether they could be cleaved by S2P in cells treated with BFA and the S1P inhibitor AEBSF. These ATF6 variants all retain transmembrane and cytoplasmic domain as well as S1P and S2P cleavage sites but have different lengths of their luminal domains (Fig. 4A–E). Reconstitution of the two-step cleavage in the ER using BFA allowed the deletion variants to be tested for S1P and S2P processing, as some of these variants cannot move to the Golgi in response to ER stress because of the loss of Golgi localization signals (6). The addition of the S1P inhibitor AEBSF showed that the deletion of LD5 and LD6 did not affect the requirement for prior S1P cleavage (Fig. 4B and C). However, further deletion of LD4 in ATF6-(1\text{–}475) resulted in a protein that could be cleaved by S2P independent of S1P proteolysis (Fig. 4D). The C-terminal deletion to aa 450 gave a similar result (Fig. 4E).

These results suggest that the LD4 domain (aa 476\text{–}500) might contain specific sequences essential for blocking S2P digestion. To test this possibility, we made an internal deletion of the LD4 region in the context of full-length ATF6. However, this variant, ATF6-(1\text{–}475)/H9004\text{–}500, was resistant to S2P digestion when S1P activity was blocked with AEBSF, suggesting that LD4 is not required for the S2P avoidance property (Fig. 4F). It is possible that multiple sequences exist in the luminal domain of ATF6 that can specifically block S2P or, alternatively, that the S2P blocking activity is not due to a specific sequence but rather to the size of the luminal domain of ATF6. We tested for the latter model by replacing the luminal domain of ATF6 with the domains from two irrelevant proteins: 1) immunoglobulin heavy chain (Ig HC) and 2) IRE1\text{}/H9251. Ig HC is a secreted protein that is normally processed in the ER, whereas IRE1\text{}/H9251 is a constitutive ER transmembrane protein (22, 23). Because neither of these proteins is a substrate of S1P or S2P, it is highly unlikely that these proteins contain any specific sequences that interfere with S2P activity. When Ig HC was fused to ATF6-(1\text{–}430), the resultant chimera ATF6-HC was still cleaved in response to BFA (Fig. 4G). This digestion was blocked by
A specific interaction was found between the substrate RseA and the intramembrane protease YaeL (15). To test whether there was a functional interaction between the luminal domain of ATF6 and S2P, we overexpressed the isolated luminal domain of ATF6 and tested whether it could function as a dominant negative form to block S2P proteolysis of ATF6. We reasoned that, if there is specific association between S2P and the luminal domain of ATF6, overexpression of the latter should interact with and inhibit S2P, such that ATF6 proteolysis would be blocked. We fused the signal peptide of Ig HC to the luminal domain of ATF6 to target it to the ER and included a C-terminal epitope tag for detection (Fig. 5A). This construct resulted in the expression of a 45-kDa protein that was localized to the ER (Fig. 5B and data not shown). ATF6-(1–430) was expressed with the soluble luminal domain of ATF6 and induced for S2P digestion with BFA. We estimate from immunoblots that the luminal domain was expressed three times more strongly than ATF6-(1–430) (data not shown). We found that the overexpression of the luminal domain of ATF6 did not affect the processing of ATF6-(1–430) (Fig. 5C). These results further suggest that there is no specific interaction between the luminal domain of ATF6 and S2P that could account for inhibition of S2P activity.

**DISCUSSION**

We found that the dependence of S2P proteolysis of ATF6 on prior site-1 cleavage is due to the size of the luminal domain of substrate that blocks S2P cleavage until it is removed by S1P digestion. We ruled out an alternative model that S2P is localized in a different cellular compartment by showing that it co-localized with S1P in the Golgi. In addition, the precise C terminus created by S1P cleavage was not required for S2P recognition since variants with longer luminal domains were still efficiently cleaved. Further, there does not appear to be a specific sequence in the luminal domain of ATF6 that blocks S2P cleavage since the internal deletion of a sequence identified by C-terminal truncation did not affect inhibition of S2P digestion. More importantly, the replacement of the luminal domain of ATF6 with two irrelevant proteins reconstituted the inhibition of S2P digestion, suggesting that it is the size rather than the specificity of the sequence that is responsible.

This is markedly different from the two-step processing of RseA in a bacterial extracytoplasmic stress response. In this case, the luminal domain of RseA was found to contain two glutamine-rich regions that interact with the intramembrane protease YaeL PDZ domain and thereby silence the enzymatic activity of YaeL (15). However, our result with ATF6 is quite similar to that of presenilin digestion of Notch in *Drosophila*. The cleavage of Notch by presenilin was blocked when the extracellular domain was -300 amino acids and digested efficiently when it was -50 amino acids long (14). Similarly, for ATF6 we found that 100 amino acids or more in the luminal domain blocked S2P digestion while 75 amino acids or less did not.

The size of the luminal domain cannot account for the requirement of S2P cleavage of SREBP for prior S1P digestion, because SREBP does not contain a large luminal domain. SREBP contains two transmembrane domains, such that is has a short luminal loop of 30 amino acids between the two transmembrane domains with both the N and C termini in the cytosol (20). The S2P cleavage site is within the first transmembrane helix of SREBP, whereas the S1P site is in the luminal loop. S1P cleavage removes the attachment of the second transmembrane and C-terminal cytoplasmic domains. This raises the possibility that the second transmembrane region of SREBP may play a similar role as the luminal domain of ATF6 in preventing S2P proteolysis. This provides an alternative mechanism to inhibition by a large luminal domain and may reflect the strategy used by SREBP.

How is this inhibition achieved? The simplest explanation is

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**FIG. 5.** Overexpression of the luminal domain of ATF6 does not attenuate ATF6 proteolysis. A, diagram of a construct encoding the luminal domain of ATF6. The signal peptide of immunoglobulin heavy chain was fused at the N terminus of the luminal domain of ATF6, and three tandem copies of the FLAG epitope were added at the C terminus. B, HeLa cells were transfected with either ATF6 LD-3×FLAG (lane 2) or an empty vector (lane 1). Whole cell lysates were immunoblotted with anti-FLAG antibodies. C, ATF6-(1–430) was co-expressed in HEK293 cells with either ATF6 LD-3×FLAG or empty vector. Cells were either untreated or treated with 5 μg/ml BFA for 1 h to induce S2P proteolysis. The position of the mature (M) form of ATF6 is indicated.
that a bulky luminal domain or a second transmembrane domain blocks access of the protease to the substrate site in the membrane. Another I-CLIP, presenilin, is the core of the γ-secretase complex that also requires nicastrin, APH-1, and PEN-2 for activity (24). The activity of S2P in vitro has yet to be demonstrated, and it is likely that it also requires additional components. In addition, S2P is a polytopic membrane protein with at least four transmembrane domains (12). Therefore, it is easy to envision that the S2P catalytic center is surrounded by multiple transmembrane helices, leaving limited space for substrate entry, such that a second transmembrane domain or a bulky luminal domain in a substrate might block S2P access to its substrate cleavage sites. As a result, previous juxtamembrane proteolysis is required to convert the substrates into a form accessible to the protease. An alternative model that has been proposed for SREBP is that the removal of its second transmembrane domain by S1P allows movement of the substrate site within the membrane (18). This type of mechanism seems less likely with ATF6 where the difference of a luminal form accessible to the protease. An alternative model that has been proposed is that digestion by S2P is blocked by regions of 100 versus 75 amino acids determined S2P cleavage. This difference would have to differentially affect the conformation or position of the proximal transmembrane domain.

However, it is notable that several intramembrane proteases require helix-breaking residues within the transmembrane regions of their substrates for cleavage. The mutation of the helix-breaking asparagine and proline residues within ATF6 and SREBP blocked S2P digestion (4, 18). Similar results were also obtained with signal peptide peptidase and the Drosophila protease Rhomboid that digests Spitz (25, 26). Interestingly, the positions of these residues within the membrane were found to be rather flexible, such that shifting their positions did not affect intramembrane proteolysis (18, 25, 26). Hence, it was proposed that it might be the general conformation, rather than a specific recognition motif, that determines intramembrane proteolysis. Helix-breaking residues may create extended structures accessible for intramembrane proteases, whereas a lack of these residues would result in rigid helical conformations resistant to digestion (25, 26).

In general, the I-CLIP proteases require prior substrate cleavage for digestion. The exception is Rhomboid, which can cleave full-length Spitz (1, 26). The main difference of Rhomboid from the other proteases is that its function is to release the extracytoplasmic domain of its substrate from the membrane, whereas the other factors cause the release of intracellular domains (1). It has been proposed that digestion by Rhomboid is more specific than the other I-CLIP proteases, such that it does not require the specificity provided by the first-step protease (26). It is noteworthy that the Rhomboid substrate Spitz contains a cytoplasmic region of only ~70 amino acids and a luminal domain of ~120 amino acids, such that these sequences may be too short to inhibit Rhomboid activity.

In contrast to Rhomboid, the proteases signal peptide peptidase, presenilin, YaeL, and S2P all require prior cleavage of their substrates (1). Presenilin is part of the γ-secretase complex that digests amyloid precursor protein as well as Notch. Digestion by γ-secretase and S2P is blocked by regions of 100–300 amino acids in the luminal domains of Notch and ATF6. It will be interesting to determine whether this rule holds up with other substrates such as amyloid precursor protein or other intramembrane proteases such as signal peptide peptidase.

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