Structural Requirements for Sterol Regulatory Element-binding Protein (SREBP) Cleavage in Fission Yeast

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Background: Yeast sterol regulatory element-binding proteins (SREBPs) are proteolytically activated by a mechanism distinct from that of mammals.

Results: Site-directed mutagenesis studies define structural requirements for yeast SREBP cleavage.

Conclusion: Yeast SREBP cleavage requires a novel conserved glycine-leucine motif.

Significance: Understanding SREBP structural requirements aids mechanistic dissection of SREBP cleavage essential for fungal pathogenesis.

Sterol regulatory element-binding proteins (SREBPs) are central regulators of cellular lipid synthesis and homeostasis. Mammalian SREBPs are proteolytically activated and liberated from the membrane by Golgi Site-1 and Site-2 proteases. Fission yeast SREBPs, Sre1 and Sre2, employ a different mechanism that genetically requires the Golgi Dsc E3 ligase complex for cleavage activation. Here, we established Sre2 as a model to define structural requirements for SREBP cleavage. We showed that Sre2 cleavage does not require the N-terminal basic helix-loop-helix zipper transcription factor domain, thus separating cleavage of Sre2 from its transcription factor function. From a mutagenesis screen of 94 C-terminal residues of Sre2, we isolated 15 residues required for cleavage and further identified a glycine-leucine sequence required for Sre2 cleavage. Importantly, the glycine-leucine sequence is located at a conserved distance before the first transmembrane segment of both Sre1 and Sre2 and cleavage occurs in between this sequence and the membrane. Bioinformatic analysis revealed a broad conservation of this novel glycine-leucine motif in SREBP homologs of ascomycete fungi, including the opportunistic human pathogen Aspergillus fumigatus where SREBP is required for virulence. Consistent with this, the sequence was also required for cleavage of the oxygen-responsive transcription factor Sre1 and adaptation to hypoxia, demonstrating functional conservation of this cleavage recognition motif. These cleavage mutants will aid identification of the fungal SREBP protease and facilitate functional dissection of the Dsc E3 ligase required for SREBP activation and fungal pathogenesis.

Cellular lipid synthesis and homeostasis are centrally regulated by sterol regulatory element-binding protein (SREBP)2 transcription factors (1, 2). These membrane-bound transcription factors contain two transmembrane segments and are inserted into the endoplasmic reticulum (ER) in a hairpin orientation with the N and C termini projecting into the cytosol (2). The SREBP N terminus contains a basic-helix-loop-helix zipper (bHLH-zip) transcription factor domain that is required for DNA binding and transcriptional activation of its target genes required for cholesterol and fatty acid synthesis (2). In mammalian cells following sterol-regulated transport to the Golgi, SREBPs are activated through sequential cleavage by the Golgi-resident Site-1 protease and Site-2 protease. Specifically, Site-1 protease is a subtilisin/kexin-like, serine protease that cleaves in the SREBP luminal loop, splitting the molecule in half. This first cleavage event enables the Site-2 protease zinc metalloprotease to cleave within the first transmembrane segment (TM1) and release the soluble SREBP N-terminal transcription factor from the membrane (3). In mammals, Site-2 protease is essential for membrane release and SREBP activity.

Fungal SREBPs have been characterized in Schizosaccharomyces pombe and the human pathogens Cryptococcus neoformans and Aspergillus fumigatus (4–9). Collectively, these studies revealed that fungal SREBPs are oxygen-responsive transcription factors required for adaptation to hypoxia and fungal pathogenesis (10). Fission yeast S. pombe contains two SREBP homologs: Sre1 and Sre2 (4). Sre1 is an oxygen-responsive transcription factor that is cleaved under low oxygen to promote adaptation to hypoxia (4). In contrast, cleavage of the less well characterized Sre2 occurs in the presence of oxygen and is unregulated. This feature of Sre2 makes it a useful model to study structural requirements for cleavage under routine cell culture conditions rather than low oxygen (11, 12).

Fission yeast lacks an identifiable Site-2 protease homolog, suggesting a different mechanism for SREBP cleavage (2). Previously, we showed that Sre1 is cleaved at a cytosolic position, instead of within TM1, consistent with Site-2 protease-inde-
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Yeast SREBP cleavage requires conserved glycine-leucine motif in fungal SREBP cleavage activation, provide tools for dissecting the mechanism of SREBP cleavage, and identify mutant alleles of sre1 at the his3+ locus.

Low Oxygen Cell Culture—For Sre1 hypoxic cleavage assays, cells growing exponentially in YPS medium were collected by centrifugation. Oxygenated medium was removed by aspiration, and cells were resuspended in deoxygenated YPS medium under anaerobic conditions inside a Ruskinn In Vivo 2400 hypoxic work station (Biotrace, Inc.). Anaerobic conditions were achieved in the work station using 10% hydrogen gas balanced with nitrogen in the presence of palladium catalyst. YPS medium was deoxygenated by preincubation for >24 h in the hypoxic work station. After resuspension, cultures were agitated at 30 °C, harvested by centrifugation, washed with water, and frozen as cell pellets in liquid nitrogen.

Cleavage Assays—For Sre1 and Sre2 cleavage assays, whole cell lysates were prepared for immunoblotting analysis. Protein preparation and immunoblotting for S. pombe experiments were described previously (4). For Sre1 and Sre2, whole cell lysates were extracted and treated with alkaline phosphatase as described previously (4). For Sre2 model substrate, anti-FLAG M2 antibody was used to detect the precursor (P) and the cleaved N-terminal nuclear form (N). For Sre2-GFP, anti-Sre2 polyclonal antibody was used to detect the precursor (P) and cleaved N-terminal nuclear form (N).

Fluorescence Microscopy—A plasmid expressing GFP-sre2 from the thiamine repressible nmt* promoter (pAH230) was described previously (12). Cleavage mutants were generated in this plasmid using QuikChange II XL mutagenesis. Live cells were imaged on 2% agarose pads using a Zeiss Axioskop microscope equipped with fluorescence and Nomarski optics (Zeiss). Images were captured using a Photometrics Cool Snap EZ CCD camera and IP Lab Spectrum software (Biovision Technologies, Inc.).

Mutagenesis Screen of Sre2 Model Substrate—Sre2 model substrate was expressed from a plasmid containing a truncated form of Sre2 (aa 423–793), tagged with a 3×FLAG epitope at its N terminus, under control of the constitutive CaMV promoter. pCaMV mutant plasmids listed in Table 1 and Fig. 3 were generated by mutation of the appropriate codons using QuikChange II XL mutagenesis. In the model substrate, 94 aa at the distal end of Sre2 (aa 676–793), which spans the conserved glycine-leucine motif and two transmembrane segments, were analyzed for cleavage. These mutants were first mutated in groups of doublets or triplets. For the mutants that demonstrated cleavage defects, individual residues were mutated from each group to analyze the contribution to Sre2 cleavage defect. Cleavage defects in model substrate were confirmed by gener-
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**Minimal Sequence Required for Sre2 Cleavage**—Sre2 is a 793-aa amino acid protein with two TM segments (aa 714–737 and 749–771) (Fig. 1A). To determine which region of Sre2 is required for Dsc-dependent cleavage, we generated serial N-terminal truncations of Sre2 and assayed cleavage of these mutants in whole cell extracts by immunoblotting (Fig. 1A). To monitor Sre2 cleavage events, we inserted a 3×FLAG epitope at the N terminus of Sre2 truncations, thereby allowing us to distinguish Sre2 precursor from Sre2 N-terminal cleaved form. Deletion of the N-terminal half of Sre2, aa 1–422, had no effect on cleavage. N-terminal truncated Sre2 (aa 423–793) showed a cleavage pattern similar to full-length Sre2 (12), with the majority of Sre2 in the cleaved form at steady state (Fig. 1B, lane 1). Importantly, this cleavage required Dsc1 (Fig. 1B, lane 2), the Golgi E3 ligase required for the cleavage activation of both Sre1 and Sre2 precursors (12). This result indicated that cleavage does not require Sre2 aa 1–422.

Further Sre2 truncations across the bHLH-zip domain (aa 426–516) demonstrated that this domain is also not required for cleavage (Fig. 1B, lanes 3 and 5), despite the requirement of this domain for DNA binding and transcription factor activity. Thus, the functions of Sre2 as a transcription factor and a substrate for Dsc-dependent cleavage are separable. Finally, Sre2 failed to cleave when we truncated Sre2 to aa 588 and the Sre2 precursor migrated at the same position in the presence or absence of dsc1 (Fig. 1B, lanes 7 and 8). Taken together, these results suggest that aa 1–522 including the bHLH-zip domain of Sre2 are not required for cleavage. However, sequences in the C-terminal portion of Sre2 are important for cleavage.

**Cleavage of Sre2 Occurs in the Cytosol**—Dsc-dependent cleavage of the oxygen-regulated Sre1 transcription factor occurs in the cytosol at a position ~10 aa before the TM1 (12). Next, we mapped the cleavage site for Sre2. Because Sre2 cleavage occurs at a site close to its C terminus, Sre2 precursor and its cleaved nuclear form are not readily resolved by SDS-PAGE. Therefore, we chose to use an Sre2 model substrate, Sre2 aa 423–793 (hereafter, Sre2-MS), that is efficiently cleaved in a Dsc-dependent manner to analyze Sre2 sequences required for cleavage (Fig. 1B, lanes 1 and 2). We verified that the N terminus of GFP-Sre2 aa 423–793 translocates to the nucleus, demonstrating that Sre2-MS is functionally imported after Dsc-dependent cleavage (data not shown). These data are consistent with studies on mammalian SREBP-2 showing that the bHLH-zip domain functions as a nuclear import signal (21).

Cleavage of mammalian SREBPs by Site-2 protease occurs within TM1 between a leucine and cysteine residue, both of which are not present in Sre2 (22). To estimate the position of Sre2 cleavage, we generated two C-terminal truncations of Sre2-MS encoding aa 423–697 and 423–712. These two size standards truncated Sre2-MS at cytosolic positions prior to the TM1 (Fig. 1A). Cleaved Sre2-MS migrated between the two C-terminal truncations (Fig. 2A, lane 4). Thus, Sre2 is cleaved between aa 697 and 712 in the cytosol, consistent with cytosolic cleavage of Sre1 (12). Notably, the cytosolic cleavage sites of Sre1 and Sre2 are equidistant (~10 amino acids) from their respective first transmembrane segments. These results reinforce the observation that SREBP cleavage in *S. pombe* is mechanistically distinct from that in mammals, whereby cleavage occurs within TM1.

**Cleavage of Sre2 Model Substrate Requires Dsc E3 Ligase Complex**—In *S. pombe*, Cdc48 and E2 Enzyme Ubc4—Cleavage of Sre1 and Sre2 requires each of the subunits of the Golgi Dsc E3 ligase complex Dsc1 through Dsc5, AAA-ATPase Cdc48, and the E2 ubiquitin-conjugating enzyme Ubc4 (11, 12). To investigate
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whether cleavage requirements of Sre2-MS parallel those of Sre1 and Sre2, we assayed cleavage of Sre2-MS in cells lacking each of these components. We found that Dsc1–Dsc4 were strictly required for Sre2-MS cleavage (Fig. 2A). In dsc5Δ cells, a small amount of Sre2-MS cleaved form was detectable (Fig. 2B, lane 6), consistent with partial requirement of dsc5 for cleavage of full-length Sre2 precursor (11). cdc48Δ contains a mutation (E325K) that lies in the Walker B motif in the AAA-ATPase domain of Cdc48 (11). This mutation causes a complete block of Sre2 cleavage compared with other cdc48 mutant alleles identified in a previous genetic screen, such as cdc48–2 (A586V) and cdc48–3 (E731K) in the D2 domain of Cdc48, that cause a severe impairment but not complete block (11). Consistent with this, Sre2-MS cleavage was blocked completely in cdc48–4 (Fig. 2B, lane 7) but not in cdc48–2 and cdc48–3 (data not shown). Finally, a temperature-sensitive allele ubc4–P61S of the essential E2 enzyme Ubc4 was utilized to test its role in Sre2-MS cleavage. Upon shifting to nonpermissive temperature to block Ubc4 function, the precursor form of Sre2-MS accumulated in ubc4–P61S, but not wild-type cells (Fig. 2C). This result indicated that Sre2-MS cleavage requires Ubc4. Taken together, these results demonstrate that Sre2-MS shares all functional requirements for Dsc-dependent cleavage with full-length Sre2 (aa 1–793) and Sre1 (11, 12) and thus is a model substrate for further mutational studies.

Site-directed Mutagenesis Screen of Sre2 Model Substrate—
To understand comprehensively the requirements for Sre2 cleavage, we set out to screen for cleavage defects by site-directed scanning mutagenesis of Sre2-MS. Based on protein secondary structure prediction programs for structured regions and given that cleavage of both Sre1 and Sre2 occurs close to the first transmembrane segment, we focused our studies on the C-terminal 117 amino acids of Sre2-MS, mutating residues 677–793 (Fig. 3A). First, we tested a total of 94 amino acid residues by mutating amino acids either in single, double, or triple mutants (supplemental Table 1). We chose not to mutate hydrophobic residues in the transmembrane segments as these would likely not be well expressed. Mutants were grouped into three classes according to cleavage efficiency (the ratio of processed form (N) to precursor form (P)) (Table 1). 51 mutants showed normal cleavage (N/P), 28 showed a defect in cleavage (N>P), and 15 showed a robust block in cleavage (P>N). Examples of each class are shown in Fig. 3B. For double or triple mutants that demonstrated a robust cleavage block (P>N), we further tested cleavage requirements by mutating individual amino acids to alanine for cytosolic residues and to leucine for residues within the transmembrane segments. For selective residues that are structurally similar to alanine or leucine, we mutated amino acids to charged residues such as glutamate or threonine. Fig. 3A and Table 1 summarize the results of the Sre2-MS mutagenesis screen.

From the screen, we identified a short, 7-amino acid glycine-leucine region required for Sre2 cleavage (aa 678–684) (Fig. 3A). Interestingly, this glycine-leucine stretch is present in Sre1 (Fig. 4A) and is roughly equidistant from the first transmembrane segment in both Sre1 and Sre2 (27 and 29 amino acids, respectively). These observations suggested a role for the glycine-leucine region in Sre2 cleavage. To investigate whether this conserved sequence (aa 678–684) was required for cleavage, we assayed cleavage of single residue mutants. Notably, mutating 5 of 7 residues blocked cleavage when tested individually (Fig. 4B, lanes 3–7).

In addition, we identified 10 other single residues required for cleavage (Fig. 3A). Two residues, Met-704 and Ser-705, are located ~10 amino acids before TM1 close to the cleavage site (Fig. 4C, lanes 3–12). We identified only a single residue, Lys-743, required for cleavage in the short endoplasmic reticulum luminal loop of Sre2. Mutation of five residues in the TM2 blocked cleavage (Glu-755, Ser-765, Pro-767, Asp-770, Trp-771) whereas none in TM1 was absolutely required (Fig. 3A). Finally, two charged residues in the cytosolic Sre2 C-terminal tail (Arg-778 and Glu-788) were also required for cleavage. In total, 15 C-terminal residues were found to be essential for Sre2-MS cleavage (Fig. 3A).

Validation of Sequence Requirements in Full-length Sre2—To independently test and verify the cleavage requirement for residues identified in the Sre2-MS screen, we generated each of the cleavage mutants in full-length GFP-Sre2. Fusion of GFP to the C terminus allows clear discrimination between the precursor
(P) and cleaved nuclear (N) forms of Sre2 (12). Wild-type GFP-Sre2 was cleaved constitutively to generate Sre2N, and cleavage was blocked in dsc1/Δ cells (Fig. 5A, lanes 1 and 2). Mutation of each of the five glycine-leucine region residues blocks cleavage of full-length GFP-Sre2 (Fig. 5A, lanes 3–7), emphasizing the importance of this sequence. Indeed, we observed a complete cleavage block for each of the additional 10 residues identified using Sre2-MS, examples of which are shown in Fig. 5B. These results validate Sre2 aa 423–793 as a model substrate and confirm the importance of these 15 residues for cleavage of full-length Sre2.

As a complementary approach to test the cleavage requirements for individual Sre2 residues, we assayed localization of different GFP-Sre2 fusion proteins in cells lacking endogenous Sre2. GFP fused to the N terminus of wild-type Sre2 translocated to the nucleus after its release from the membrane (left panel of Fig. 5C). Failure to cleave GFP-Sre2 in dsc1/Δ cells caused GFP-Sre2 to localize in punctate structures (middle panel of Fig. 5C) (12). Consistent with a requirement for Dsc-dependent cleavage, GFP-Sre2 Gly-683 containing a mutation in the glycine-leucine stretch also localized to punctate structures (right panel of Fig. 5C). We tested all of the 15 individual cleavage mutants in this GFP-Sre2 assay and each failed to show nuclear localization equivalent to wild-type GFP-Sre2 (data not shown). Combined with the full-length GFP-Sre2 cleavage assays, these data demonstrate that full-length Sre2 cleavage requires each of the 15 residues isolated using Sre2-MS, showing the physiological importance of these sequences.

Bioinformatic Analysis Reveals Wide Conservation of Glycine-Leucine Motif in Ascomycete Fungi—Our mutagenesis screen identified a glycine-leucine sequence in Sre2 (aa 678–684) that is required for cleavage and conserved in Sre1 (Fig. 4A). Interestingly, the distance of this sequence from the first transmembrane segment (H11011) 30 amino acids) is also conserved between Sre2 and Sre1. To investigate whether the glycine-leucine region is conserved beyond S. pombe, we searched for glycine-leucine sequences in SREBPs ranging from fungi to mammals using a defined set of bioinformatic criteria. Given the short length of the conserved sequence, parameter definition was critical for the motif search and subsequent consensus motif generation. First, we isolated SREBPs from all species by sequence similarity search. We defined SREBPs by the presence of a specific tyrosine residue in the first helix of bHLH transcription factors. For each SREBP, we predicted coordinates for transmembrane segments using transmembrane prediction software, and we used only SREBPs that contained a predicted TM for subsequent analysis. Given that the glycine-leucine sequence is located at a conserved distance from TM1s of Sre1 and Sre2, we selected a 50-amino acid stretch that is centered 55 amino acids from the beginning of TM1 for each SREBP. We then performed a sequence alignment using all isolated 50-amino acid sequences. In this way, we identified a consensus
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TABLE 1

| Sre2 model substrate mutants screened | Cleavage of Sre2 model substrate mutants | Normal (N>P) |
|--------------------------------------|--------------------------------------|--------------|
| Block (P>N) | Partial (P>N) | Normal (N>P) |
| L678T | N679E | V677T |
| G680E | E685A | V682T |
| L681T | T686A | T691A/N692A |
| G683E | V687A/H688A | G697A |
| L684T | M689A/L690A | S699A |
| M704A | P693A | R701A |
| S705A | D694A | F702A/S703A |
| K743R | D695A | V706A/L707A |
| E755L | S698A | S710A/P711A |
| S765L | N700A | S712A/L713A |
| P767L | P708A | H714L/S715L |
| D770L | I709A | H714L/S715L |
| W771L | T718L/F719L | F726L |
| R778A | S751L | C728L/F729L |
| E788A | R753L | T736L |
| Y789A | L783A | K743R |
| N776A | V757L | L783A |
| L777A | F758L | L783A |
| L781A | F764L | L783A |
| L787A | G772A | Y774A |
| L775A | G779A | G779A |
| K780R | R743R | G779A |
| L782A | E794A/L785A/N786A | S789A |
| L783A | S790A | G790A/V791A |
| L784A | T793A | T793A |

sequence corresponding to the glycine-leucine motif (Fig. 6A). We identified the glycine-leucine motif in all ascomycete fungi analyzed, including pathogenic fungi A. fumigatus and Magnaporthe oryzae, but not basidiomycete fungi, like C. neoformans (supplemental Table 2). Interestingly, C. neoformans utilizes Site-2 protease to cleave and activate SREBP like mammalian cells (23). Collectively, this bioinformatic analysis identified a conserved glycine-leucine motif present in ascomycete fungi that lack Site-2 protease, further supporting a role for this sequence in SREBP cleavage.

Sre1 Cleavage Requires Glycine-Leucine Motif—Having identified this conserved glycine-leucine motif, we next tested whether motif function is conserved between SREBPs. Fission yeast strains were generated that expressed wild-type or sre1 glycine-leucine motif mutants at the heterologous his3 locus (18). We cultured cells under low oxygen and assayed Sre1 cleavage by immunoblotting. Wild-type Sre1 precursor was cleaved to generate active Sre1N under hypoxia (top, middle, and bottom panels of Fig. 6B, lanes 1–4). Induction of Sre1 expressed from the his3 locus was reduced compared with wild-type cells, perhaps due to the absence of DNA elements required for key positive feedback regulation (4, 24, 25). As a control, we tested mutation of Sre1 N408E in the 7-amino acid conserved motif, corresponding to the second position in the motif that was not required for cleavage of Sre2 (aa 679) (Fig. 6A). In agreement, mutation of this residue did not affect cleavage of Sre1 (top panel of Fig. 6B, lanes 5–8). Then, we tested four of five required residues in the motif for Sre1 cleavage. Notably, mutation of glycine at the third position (Gly-409), methionine at the fourth position (Met-410), glycine at the sixth position (Gly-412), and leucine at the seventh position (Leu-413) blocked Sre1 cleavage under low oxygen (middle and bottom panels of Fig. 6B, lanes 5–12). These results were consistent with Sre2-MS, showing cleavage defects at the third, fourth, sixth, and seventh positions of the motif (Sre2 aa 678, 680, 683, and 684) (Fig. 6A). As expected, deletion of the E3 ligase dsc1 abolished Sre1 cleavage (middle panel of Fig. 6B, lane 13). In these experiments, the cleavage machinery functioned normally insomuch as endogenous Sre2 cleavage was normal (data not shown).

As an independent test, we assayed the ability of these sre1 mutants to grow in the presence of cobalt chloride, a hypoxia mimetic (6, 12). Wild-type cells, but not sre1Δ cells, grow under low oxygen (4). If Sre1 mutants are cleavage-defective, we expect to observe reduced growth on cobalt chloride, reminiscent of growth defects in sre1Δ and dsc1Δ cells. Consistent with results from the cleavage assays in Fig. 6B, Sre1 mutants blocked for cleavage failed to support wild-type growth on
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-FIGURE 5. Mutants are defective for cleavage of full-length Sre2. A, Western blot probed with anti-Sre2 IgG of lysates from the indicated sre2Δ strains expressing either full-length wild-type GFP-Sre2 or glycine-leucine motif mutants. B, Western blot probed with anti-Sre2 IgG of lysates from the indicated sre2Δ strains expressing either full-length wild-type GFP-Sre2 or cleavage-defective mutants. P and N, precursor and cleaved nuclear forms of GFP-Sre2, respectively. C, indicated sre2Δ strains expressing either wild-type GFP-Sre2 or GFP-Sre2 mutant grown in minimal medium lacking leucine and thiamine for 20 h to induce expression and imaged by fluorescence microscopy.

cobalt chloride (Fig. 6C), and growth defects correlated with the severity of observed Sre1 cleavage defects (Fig. 6B). These results demonstrate that the glycine-leucine motif is required for Sre1 cleavage, and together with the bioinformatic data suggest that this motif has a conserved function in SREBP cleavage in ascomycete fungi.

DISCUSSION

Genetic studies revealed requirements for dsc1 through dsc6 in the cleavage activation of the yeast SREBP transcription factors Sre1 and Sre2 (11, 12). Dsc1 through Dsc5 constitute the Dsc E3 ligase, a stable Golgi membrane complex containing the RING domain E3 ubiquitin ligase Dsc1 (11). dsc6 codes for Cdc48, an AAA-ATPase that binds to the Dsc E3 ligase through the ubiquitin regulatory X domain of Dsc5 (11). Previous studies indicate that the mechanism of cleavage is the same for Sre1 and Sre2 (11, 12), except that ER exit of Sre1 is regulated by sterols and oxygen and Sre2 exits the ER constitutively (4, 25). Our working model for yeast SREBP (Sre1 and Sre2) cleavage is as follows: (i) SREBP moves from the ER to Golgi; (ii) SREBP binds to the Dsc E3 ligase; (iii) SREBP is ubiquitinated by the E2 ubiquitin-conjugating enzyme Ubc4 and E3 ligase Dsc1; (iv) SREBP is subsequently cleaved by an unidentified protease releasing the N-terminal transcription factor domain from the membrane.

To develop tools that will allow us to test this model and dissect the mechanism of SREBP cleavage, we investigated the structural requirements for SREBP cleavage. We focused initially on Sre2 because its cleavage is constitutive, allowing us to study sequences required for cleavage under routine cell culture conditions rather than having to induce cleavage under hypoxia. Truncation analysis revealed that Sre2 cleavage does not require its bHLH-zip domain and demonstrated that a minimal substrate of 271 amino acids is still cleaved (Fig. 1). This result indicates that the functions of Sre2 as a transcription factor and a substrate for Dsc-dependent cleavage are separable. Using a truncated Sre2 model substrate, we identified 15 residues required for cleavage of full-length Sre2 and uncovered a novel SREBP cleavage motif. We discuss these mutants in light of our current understanding of SREBP cleavage in S. pombe and mammals.

Mutants blocked for Sre2 cleavage could be defective in any of the four steps outlined in our working model for SREBP cleavage. Sre2 cytosolic mutations could disrupt binding to COPII proteins required for sorting into vesicles and ER exit (26). However, none of the 15 Sre2 mutants localized to the ER when tested in either the GFP-Sre2 (Fig. 5C) or GFP-Sre2-MS localization studies (data not shown), suggesting that ER exit is normal in the mutants. In addition, Sre2 mutants showed wild-type expression (Fig. 4, B and C), suggesting that the proteins are properly folded and unlikely to be substrates for ER retention and ER-associated degradation (27). Determinants required for ER exit may be located between aa 523 and 676, a region required for cleavage (Fig. 1B), but not subjected to site-directed mutagenesis.

Function of Conserved Glycine-Leucine Motif in SREBP Cleavage—Several lines of evidence suggest that the glycine-leucine motif is a key determinant for Dsc-dependent SREBP cleavage. First, the glycine-leucine motif is located at a con...
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FIGURE 6. Glycine-leucine motif is broadly conserved in fungi and functionally conserved in Sre1. A, consensus SREBP glycine-leucine motif in fungi. Consensus sequence logo was generated by aligning 50-amino acid stretches that were isolated and centered at 55 amino acids before the first predicted TM of fungal SREBPs (see “Experimental Procedures” for details). Residues required for Sre2 cleavage are boxed. B, Western blot probed with anti-Sre1 IgG of lysates from the indicated full-length wild-type Sre1 and glycine-leucine motif mutants expressed from the chromosome. Cells were grown in rich medium for the indicated time in the absence of oxygen. Sre1 N408E is used as a control, corresponding to a residue with normal cleavage in Sre2. P and N, precursor and cleaved nuclear forms of Sre1, respectively. C, growth of wild-type, sre1Δ, dsc1Δ, and indicated sre1 mutants on YES-rich medium containing 1.6 mM cobalt chloride. 5x and 1x indicate 25,000 and 5,000 cells, respectively.

Protein secondary structure software predicts this novel glycine-leucine motif folds either as α-helix or β-strand. This motif might represent a hydrophobic interface for interaction with the Dsc E3 ligase or the unidentified fungal SREBP protease. Given that the motif is located at a distance (~20 amino acids) from the cleavage site, it is unlikely to participate in interactions with the protease active site. Indeed, a definitive functional assignment for this motif requires the development of additional assays for Dsc E3 ligase recognition, SREBP ubiquitination, and SREBP proteolytic cleavage.

As noted, the protease that cleaves SREBP in this system is not known. Mammalian SREBP is cleaved sequentially by the Site-1 protease after RXXL in the ER luminal loop before it can be cleaved by the Site-2 protease within TM1 to release functional transcription factor domain (28). Site-1 protease belongs to the subtilisin/kexin-like protease family, required for cleaving many proproteins to their active form (29, 30). Interestingly, we have isolated a cleavage mutant in the ER luminal loop of Sre2 at lysine 743. A charge-conservative mutation from lysine to arginine blocks Sre2 cleavage (Fig. 3). Subtilisin/kexin-like proteases typically cleave after lysine or arginine and dibasic sequences (29, 30), as evidenced by normal SREBP cleavage in mammals for the same corresponding Lys to Arg mutation (22). Further, in contrast to the cleavage of mammalian SREBP by Site-1 protease at a luminal RXXL sequence, this sequence is not conserved in Sre2 (22). More generally, proteases in the subtilisin/kexin-like family cleave after the consensus RX(R/K)R sequence, where X is any amino acid except cysteine (31). Collectively, these data argue against a Site-1 protease-like cleavage for yeast SREBP.

A Site-2 protease cleavage mechanism is also unlikely given the cystolic cleavage sites of Sre1 (12) and Sre2 (Fig. 2A). Sre2 cleavage defects at methionine 704 and serine 705 represent cleavage mutants close to the cleavage site (Fig. 3A). These mutants might interfere directly with interaction between Sre2 and the unidentified protease. Consistent with this, the presence of Site-2 protease correlates with cleavage within the membrane (10, 23), whereas the absence of Site-2 protease correlates with cystolic cleavage. Given the lack of Site-2 protease homologs in S. pombe and the broader ascomycete fungal phyllum, and no detectable cleavage within TM1, a new cleavage mechanism might be employed for ascomycete SREBPs.
Sre2 cleavage mutants are enriched in TM2 (Fig. 3A), but a close examination of other SREBP TM2s did not reveal conserved sequences. Interestingly, ubiquitination and sorting of the Pep12 membrane protein into the multivesicular body required insertion of an aspartate residue into its transmembrane segment and Tuf1, the Saccharomyces cerevisiae homolog of the Dsc E3 ligase (32). Thus, it is possible that charged residues in a transmembrane segment are a signal for substrate recognition. Sre2 cleavage mutants within TM2 that contain a polar residue, such as glutamate 755, might fail to be recognized by the Dsc1 E3 ligase through the same mechanism.

Identification of the fungal SREBP protease and further characterization of Dsc E3 ligase function will require development of new assays. Importantly, these mutants will aid the identification of the protease and future structure-function studies. For example, genetic suppression of Sre1 cleavage-defective mutants may uncover components required for different steps in the pathway.

Implications for Fungal Pathogenesis—Ascomycete fungal pathogens remain detrimental to human health and agriculture. For instance, A. fumigatus is a major cause of life-threatening infections in immunocompromised individuals (33). Notably, A. fumigatus SREBP homolog SrBA as well as Dsc E3 ligase homologs are required for pathogenesis (7, 13). Interestingly, the glycine-leucine motif is conserved in Aspergillus. In terms of agriculture, the fungal rice blast pathogen M. oryzae, which causes destructive rice diseases and crop losses worldwide (34), also contains a putative SREBP homolog and the cleavage motif. Thus, this motif may be required for SREBP cleavage activation across ascomycete fungal pathogens. Because many other pathogenic fungi contain an SREBP pathway and the conserved motif, this study provides insight into molecular underpinnings of SREBP cleavage activation that may have broad antifungal applications.

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