Regulation of transcription factor latency by receptor-activated proteolysis

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The transcription factor Stp1 is endoproteolytically processed in response to extracellular amino acids by the plasma membrane SPS (Ssy1–Ptr3–Ssy5)-sensor. Processed Stp1, lacking a cytoplasmic retention motif, enters the nucleus and induces amino acid transporter gene expression. The SPS-sensor component Ssy5 is a chymotrypsin-like protease with a Pro-domain and a catalytic domain. The Pro-domain, required for protease maturation, is autolytically cleaved from the catalytic domain but remains associated, forming an inactive protease complex that binds Stp1. Stp1 is processed only after amino acid-induced signals cause the dissociation of the inhibitory Pro-domain. Our findings demonstrate that gene expression can be controlled by regulating the enzymatic activity of an intracellular endoprotease.


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A growing number of transcription factors are known to be maintained as latent cytoplasmic factors that require proteolytic processing prior to nuclear targeting. The sterol regulatory element-binding protein is released from membranes in two successive rounds by site-specific membrane-bound proteases in a process termed regulated intramembrane proteolysis [RIP] (Brown et al. 2000). Similarly, the Drosophila NFκB factor Relish requires signal-activated and caspase-mediated processing before translocation to the nucleus (Stöven et al. 2003). In yeast, transcription factors Spt23 and Mga2 are activated via their release from C-terminal membrane anchors by a process termed regulated ubiquitin/proteasome-dependent processing [RUP] (Hoppe et al. 2000). The control of transcription factor latency by proteolytic processing appears to be a useful mechanism for regulating gene expression.

Central to understanding latent factor activation is the unambiguous identification of the responsible protease, and perhaps more importantly, defining how its proteolytic activity is regulated. In principle, metabolic signals could directly control the catalytic activity of a protease [enzymatic regulation], or control factor access to a constitutively active protease [substrate regulation]. To date, only substrate regulation has been shown to play a role in the activation of latent factors in eukaryotic cells. This raises the question: Is the enzymatic control of protease activity mechanistically incompatible with signaling processes that regulate gene expression by mobilizing latent factors?

Saccharomyces cerevisiae offers a few well-defined signaling pathways from the plasma membrane to the nucleus, many of which are involved in sensing nutrient availability and regulating nutrient uptake [Forsberg and Ljungdahl 2000b; Van Belle and André 2001]. The yeast plasma membrane amino acid receptor Ssy1 functions with two intracellular proteins, Ptr3 and Ssy5, as the fundamental components of the SPS [Ssy1–Ptr3–Ssy5]-sensing pathway [Forsberg and Ljungdahl 2001a]. This pathway up-regulates transcription of amino acid permease genes in response to extracellular amino acids by controlling the activity of two homologous transcription factors, Stp1 and Stp2 [Andréasson and Ljungdahl 2002]. Stp1 and Stp2 are expressed as latent cytoplasmic precursors. Extracellular amino acids rapidly induce the endoproteolytic removal of cytoplasmic retention motifs in a fashion strictly dependent on the SPS-sensor [Andréasson and Ljungdahl 2002, 2004; Abdel-Sater et al. 2004], the SCFGrr1 ubiquitin E3 ligase complex [Abdel-Sater et al. 2004; Andréasson and Ljungdahl 2004], and casein kinase-dependent phosphorylation [Abdel-Sater et al. 2004]. The processed forms of Stp1 and Stp2 target to the nucleus where they redundantly function to induce the expression of SPS-sensor-regulated genes [Andréasson and Ljungdahl 2002].

The SPS-sensor component Ssy5 has emerged as a candidate processing protease, since an improved sequence comparison algorithm identified weak homology with serine proteases [Abdel-Sater et al. 2004; Andréasson 2004; Poulsen et al. 2006]. Consistently, Ssy5 exhibits several hallmarks of a protease, including apparent constitutive autoproteolysis of a Pro-domain, and a predicted catalytic serine residue has been shown to be required for autolysis and Stp1 processing [Abdel-Sater et al. 2004; Andréasson 2004]. Direct biochemical evidence to support the prediction that Ssy5 is the processing protease is lacking, and the regulatory mechanisms controlling its potential proteolytic activity have not been defined. Here, we have pursued the mechanism responsible for the amino acid-induced processing of Stp1. Our findings establish that Ssy5 is the processing protease, and define the manner by which its activity is controlled.

Results and Discussion

Ssy5 interacts with Stp1

We have previously reported that the first 125 amino acid residues of Stp1 contains two sequence motifs [Fig. 1A] that place this transcription factor under SPS-sensing pathway control [Andréasson and Ljungdahl 2004]. Stp1 is excluded from the nucleus due to the presence of Region I, and the SPS-sensor-induced endoproteolytic processing of Stp1 is dependent on Region II. To directly identify the processing protease, we screened a genomic
two-hybrid library for clones expressing proteins that interact with the N-terminal fragment of Stp1. We isolated 106 clones that expressed a fusion between the vector-encoded activation domain and a truncated Ssy5 lacking the first 90 amino acids. Stp163-125 was also found to interact with full-length Ssy5. The interaction motif of Stp1 was found to be within Region II (Stp163-125, Stp163-108, and Stp163-80).

We reconstituted the physical interaction using an in vitro system. First, we tested if the Stp163-125 fragment was properly processed in vivo by expressing Stp163-125 as a fusion between a dual immunoglobulin-binding Z domain (ZZ) and GST. This sandwich construct was endopeptidolytically processed in a strict amino acid- and SSY5-dependent manner (Fig. 1B). Next, we purified ZZ-STP163-125 containing a C-terminal myc tag (ZZ-Stp163-125-myc) and the control protein (ZZ-myc) from Escherichia coli. In pull-down experiments, Sy5-GST specifically associated with ZZ-Stp163-125-myc but not with the ZZ-myc control (Fig. 1C, cf. lanes 3, 5, and 2, 4). The α-GST antibody detected two species of Sy5-GST; a weak signal in the lysates corresponding in size to full-length Sy5 (Fig. 1C, lanes 1, 6, 7), and a smaller Stp1-interacting protein corresponding in size to the conserved C-terminal protease domain of Sy5 (Fig. 1C, lanes 1, 3, 5, 6). To summarize, the Stp163-125 fragment is a substrate of the SPS-sensor-processing protease, and it physically associates with the candidate protease Sy5.

The Pro-domain is required for Ssy5 protease activity

The role of the Pro-domain of Sy5 has not been defined; however, in analogy to many other proteases the Pro-domain may mediate folding of the catalytic (Cat)-domain (Racker et al. 1993; Rawlings and Barrett 1994; Hedstrom 2002). To test this possibility we expressed Pro- and Cat-domain (Fig. 2, amino acid residues 1–381 and 382–699, respectively) under control of the endogenous SSY5 promoter, either alone or in combination, and assessed their function in an ssy5-null mutant strain using a growth-based assay. Although both domains of Sy5 were expressed and migrated at the expected molecular mass (Fig. 2, bottom panels), neither the Pro- nor Cat-domains complemented the ssy5-null mutant phenotype, even when coexpressed (Fig. 2). These findings demonstrate that the Pro-domain has a vital role in the
maturation of the protease activity of Ssy5, and indicate that its function cannot be executed in trans. Consistent with the Pro-domain facilitating Cat-domain folding, the levels of the Cat-domain were markedly higher in cells expressing full-length Ssy5-GST than in cells expressing the truncated Cat-domain fragment (Fig. 2, cf. lanes 4, 5 and 2). Misfolded proteins often exhibit increased rates of degradation.

Constitutively active Ssy5 processes Stp1 in Schizosaccharomyces pombe

Encouraged by our ability to detect a putative substrate–protease association (Fig. 1), we used heterologous expression in S. pombe to test if Ssy5 can function alone to process Stp1. S. pombe lacks any orthologs of SPS-sensor components, including Stp1 and Ssy5. STP1-‐myc was integrated in the genome of S. pombe, and functional SSY5-Flag and HA-SSY5-Flag alleles were separately integrated into this strain. Immunoblotting revealed that Ssy5 and HA-Ssy5 were both expressed and properly cleaved, although HA-Ssy5 levels were reduced in comparison to Ssy5 (Fig. 3A, top and middle panels, cf. lanes 6, 7 and 1–4). Importantly, the introduction of a HA epitope tag at the N terminus of Ssy5 [HA-Ssy5] resulted in the constitutive processing of Stp1 in S. cerevisiae, even in the absence of amino acid-induced signals [Fig. 3A, bottom panel, lane 3]. Similarly, Stp1 was efficiently processed in S. pombe expressing the constitutive HA-Ssy5 [Fig. 3A, bottom panel, lane 7], but not in control cells or cells expressing untagged Ssy5 [Fig. 3A, bottom panel, lanes 5, 6].

Our data reveal several important aspects of SPS-sensor signaling. First, the observation that both Ssy5 and HA-Ssy5 are properly cleaved in S. pombe indicates that proper folding of the Cat-domain occurs in the absence of other SPS-sensor components. Second, the fact that the insertion of an HA epitope at the extreme N terminus of Ssy5 generates a constitutively active Ssy5 protease suggests that the Pro-domain has a function in addition to facilitating the maturation of the protease. Finally, the finding that Stp1 is processed in S. pombe, which lacks the known components of the SPS-sensing pathway, provides strong support for the notion that Ssy5 is indeed the Stp1 processing protease.

Processing of Stp1 in cell-free lysates

The constitutive nature of HA-Ssy5 and our finding that processing occurs in S. pombe (Fig. 3A) raised the possibility that the Stp1 processing activity of the Cat-domain is directly regulated at the enzymatic level and not by substrate accessibility. To clearly distinguish between these two possibilities, we assessed Ssy5 activity in cell-free lysates. Stp1, carrying a C-terminal HA-tag, was purified from E. coli (Fig. 3B, lane 1), and was incubated with cell-free lysates prepared from wild-type cells, and cells harboring null mutations in SSY1, PTR3, SSY5, GRR1, or STP1 STP2 grown in amino acid-free medium or medium supplemented with leucine. Lysates from cells grown in the absence of leucine did not exhibit Stp1 processing activity (Fig. 3B, top panel, cf. lanes 1 and 2–7). In contrast, Stp1 processing activity was readily detected in lysates from wild-type or stpΔ stp2Δ cells grown in leucine-supplemented medium (Fig. 3B, bottom panel, lanes 2, 7). The processing of recombinant Stp1 did not occur in lysates from leucine-induced ssy1Δ, ptr3Δ, ssy5Δ, or grr1Δ cells (Fig. 3B, bottom panel, lanes 3–6). Thus, the Ssy5 protease activity monitored by this assay faithfully reproduced SPS-sensor signaling, and clearly demonstrates that the endoproteolytic processing of Stp1 is regulated at the enzymatic level.

The Pro-domain remains associated with the Cat-domain

Our observation that an N-terminal HA-tag perturbs normal regulation of Ssy5 activity is consistent with enzymatic regulation, and even suggests a possible mechanism. Accordingly, after facilitating folding, the Pro-domain negatively affects the proteolytic activity of the Cat-domain, perhaps by remaining tightly associated, thereby blocking access to the catalytic site. We assessed the interaction between the processed Pro- and Cat-domains by purifying C-terminally tagged Ssy5-GST from cell lysates. Cells were grown under noninducing conditions [amino acid-free medium], and Ssy5-GST was overexpressed. Coomassie staining revealed a species corresponding in size to the Pro-domain that copurified in large amounts with the GST-tagged Cat-domain [Fig. 4A,
The immunoreactive forms of epitope-tagged proteins are schematically depicted at their corresponding positions of migration. The immunoreactive forms of epitope-tagged proteins are affinity-purified from extracts from SD-grown CAY324 (syy5Δ ptrb1Δ) carrying pCA229 [Ssy5-GST] or pCA230 (HA-Ssy5-GST). Bound protein was eluted, resolved by SDS-PAGE, and either stained with Coomassie (lanes 1, 3) or immunoblotted with α-GST [lanes 3, 4] or α-HA [lanes 5, 6] antibodies. (B) Time course of leucine-induced Stp1 processing. SD-grown cultures of syy5Δ strain HKY77 [pCA204 [Stp1-myc]] carrying pFL005 (HA-Ssy5, top panel) or pCA177 [Ssy5-HA, middle and bottom panels] were induced with leucine. Immunoblot analysis of extracts from subsamples of each culture were resolved at 5-min intervals after leucine addition. The signal intensity (arbitrary units) of the immunoreactive band corresponding to the HA-tagged Pro-domain was quantified; the values normalized to the t = 0 time point are plotted. (C) Immunoblot analysis of extracts from syy1Δ [HKY84], ptr3Δ [HKY85], and grr1Δ [CAY274] strains carrying pFL005 [top panel] or pCA177 [bottom panel] grown in SD and 30 min after cells were induced with leucine. The immunoreactive forms of epitope-tagged proteins are schematically depicted at their corresponding positions of migration. Stars mark the position of unrelated cross-reacting proteins.

The presence of the HA-tag at the N terminus of Ssy5-GST resulted in slower migration of the Pro-domain species, appearing on some stained gels as a band migrating just above the C-terminal species. We confirmed that this species corresponded to the Pro-domain by immunoblotting (Fig. 4A, lane 6). During review and revision of our work, a parallel study confirmed that the Pro- and Cat-domains associate (Poulsen et al. 2006). The finding that the Pro- and Cat-domains copurify favors a model where the Pro-domain inhibits the activity of the Cat-domain. If this model is correct, the dissociation of the Pro- from the Cat-domain should be a requisite for Stp1 processing.

Ssy5 processing activity correlates with Pro-domain down-regulation

To test this model, we investigated if the steady-state levels of the Pro-domain changed upon amino acid induction. We constructed a fully functional and properly regulated Ssy5 carrying an internal HA epitope tag, localized in the Pro-domain after amino acid residue 216. Cells expressing this internally tagged HAA216-Ssy5, or C-terminally tagged Ssy5 (Ssy5-HA) were grown in amino acid-free medium and induced with leucine. During a 30-min time course the levels of the Cat-domain remained unchanged, whereas the Pro-domain signal continually diminished (Fig. 5B, top two panels, α-HA); the Pro-domain levels reduced to ~50% of the initial signal 20 min after leucine induction (Fig. 5B). Furthermore, the down-regulation of the Pro-domain correlated in time with the accumulation of processed Stp1 (Fig. 5B, third panel, α-myc). Importantly, the down-regulation of the Pro-domain required Ssy1, Ptb3, and Grr1, as it was completely inhibited in respective null mutant strains (Fig. 5C). Our finding that the levels of Pro-domain are down-regulated upon SPS-sensor induction supports the notion that activation of the catalytic domain is the consequence of the release from Pro-domain inhibition. Previously, we observed that the levels of N-terminally myc-tagged Ssy5 rapidly reduced upon leucine induction, although the significance of the signal loss was not fully appreciated at that time (Forsberg and Ljungdahl 2001a).

Model for regulation of Ssy5 activity

We have found that Ssy5 is activated in two steps (Fig. 5), and that the Pro-domain has a critical role in each step. Similar to many other proteases (Baker et al. 1993; Rawlings and Barrett 1994; Hedstrom 2002), the Pro-domain of Ssy5 facilitates the correct folding of the catalytic domain (Fig. 2). Presumably, once the Cat-domain is properly folded, the autolytic event that cleaves the Pro- from the Cat-domain occurs. The Pro-domain-dependent maturation process can be viewed as a priming event; it occurs even in the absence of signaling [no amino acids], or a functional SPS-sensing pathway (S. pombe) [Fig. 3A]. Strikingly, although not covalently attached, the two domains of Ssy5 remain associated (Fig. 4A), forming an

Figure 4. The Pro- and Cat-domains copurify, and amino acid-induced signals reduce Pro-domain levels. (A) GST fusion proteins were affinity-purified from extracts from SD-grown CAY324 (syy5Δ ptrb1Δ) carrying pCA229 [Ssy5-GST] or pCA230 (HA-Ssy5-GST). Bound protein was eluted, resolved by SDS-PAGE, and either stained with Coomassie (lanes 1, 3) or immunoblotted with α-GST [lanes 3, 4] or α-HA [lanes 5, 6] antibodies. (B) Time course of leucine-induced Stp1 processing. SD-grown cultures of syy5Δ strain HKY77 [pCA204 [Stp1-myc]] carrying pFL005 (HA-Ssy5, top panel) or pCA177 (Ssy5-HA, middle and bottom panels) were induced with leucine. Immunoblot analysis of extracts from subsamples of each culture were resolved at 5-min intervals after leucine addition. The signal intensity (arbitrary units) of the immunoreactive band corresponding to the HA-tagged Pro-domain was quantified; the values normalized to the t = 0 time point are plotted. (C) Immunoblot analysis of extracts from syy1Δ [HKY84], ptr3Δ [HKY85], and grr1Δ [CAY274] strains carrying pFL005 (top panel) or pCA177 (bottom panel) grown in SD and 30 min after cells were induced with leucine. The immunoreactive forms of epitope-tagged proteins are schematically depicted at their corresponding positions of migration. Stars mark the position of unrelated cross-reacting proteins.

Figure 5. Model of the experimentally determined two-step activation of the Ssy5 protease. Ssy5 is expressed as a full-length and inactive zymogen [pro-Ssy5] that is autolytically processed [scissors, and thus catalytically competent, but inhibited by its interaction with the Pro-domain [primed]. The plasma membrane receptor Ssy1, together with Ptb3, transduces amino acid-initiated signals, resulting in the release of Pro-domain inhibition. Stp1 and Stp2 are processed [scissors] by the active Cat-domain. The processed forms of Stp1 and Stp2, lacking N-terminal negative regulatory domains, enter the nucleus, bind relevant promoters, and activate gene expression.
 inactive protease complex that associates with Stp1 in vivo and in vitro (Fig. 1). In response to extracellular amino acids and in a manner strictly dependent on the SPS-sensor, the inhibitory Pro-domain is induced to dissociate, and thereby enables the catalytic domain to process Stp1 [Fig. 4B]. Thus, the SPS-sensing pathway regulates gene expression by directly controlling the enzymatic activity of a protease.

To our knowledge, the SPS-sensing pathway is the first signal transduction pathway described in eukaryotic cells that utilizes direct activation of a protease to mobilize latent transcription factors by proteolytic processing. We term this novel mechanism receptor-activated proteolysis, or RAP, since it requires all known components of the plasma membrane SPS–amino acid receptor complex. Other signaling pathways controlled by regulated proteolysis (i.e., RIP and RUP) rely on mediating substrate availability rather than by direct regulation of protease activity [Brown et al. 2000; Hoppe et al. 2000; Stöven et al. 2003].

In prokaryotes, a possible variant of RAP has been described [Walsh et al. 2003; Wilken et al. 2004]. The bacterial DegS protease is a component of the inner membrane. In response to binding misfolded outer membrane proteins, the receptor domain of DegS undergoes a conformational change that exposes an intrinsic protease site, which leads to α-dependent stress gene expression. However, secreted prokaryotic proteases may provide a more relevant precedent to understanding Ssy5 activation. For example, the bacterial α-lytic protease is expressed with an N-terminal Pro-domain that has a key role in enabling the catalytic domain to reach a correctly folded and active state [Silen and Agard 1989; Peters et al. 1998; Cunningham and Agard 2003]. Following autoproteolysis the Pro-domain remains strongly associated with the catalytic domain, and consequently sterically blocks the active site [Cunningham and Agard 2004]. Thus, folded and functional α-lytic protease remains inactive until it reaches an environment where the Pro-domain is removed by proteolysis.

The parallels between Ssy5 and the well-studied α-lytic protease are striking. However, in contrast to the Pro-domain of α-lytic protease, the Pro-domain of Ssy5 does not function in trans to facilitate folding of the Cat-domain. Additionally, the Pro-domain of Ssy5 is uncharacteristically large (381 vs. 166 amino acids), which may be consistent with a role either in mediating interactions with substrates Stp1 and Stp2, to respond to amino acid-induced signals, or a combination of these two activities. In conclusion, it appears that the SPS-sensing pathway includes a protease with basic mechanistic features that are utilized to restrict unconstrained proteolysis by secreted bacterial relatives. The mechanistic conservation from bacteria to yeast strongly suggests that more examples of similarly regulated proteases with key functions in eukaryotic signal transduction pathways will be found.

Materials and methods

Media, strains, and plasmids

Standard media were prepared as described previously [Andréasson and Ljungdahl 2002]. SGal or SGGal contain 2% galactose and 0.1% glucose as the sole carbon source. YES medium has been described before [Andréasson and Ljungdahl 2004].
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