A Single PXY Motif Located within the Carboxyl Terminus of Spt23p and Mga2p Mediates a Physical and Functional Interaction with Ubiquitin Ligase Rsp5p*

Received for publication, September 8, 2004
Published, JBC Papers in Press, October 5, 2004, DOI 10.1074/jbc.M410325200

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Proteasome-dependent processing of the endoplasmic reticulum localized transcription factor Spt23p of Saccharomyces cerevisiae generates its transcriptionally competent form and requires the WW domain containing Rsp5p ubiquitin ligase. Although previous studies documented an Rsp5p–Spt23p association in cells, very little is known about the nature of this interaction. We report here the identification of an imperfect type I WW domain-binding site (LPKY) within the carboxyl-terminal region of Spt23p that is required for Rsp5p binding in vitro and in vivo. Deletion of this motif abrogates Rsp5p-induced ubiquitination of Spt23p in vitro and reduces ubiquitination of the Spt23p precursor in yeast. In addition, the Spt23pLPKY mutant is inefficiently processed and is defective at up-regulating target gene (OLE1) expression in cells. Deletion of the corresponding LPKY site within Mga2p, an Spt23p homologue, also abrogates Rsp5p binding and Rsp5p-dependent ubiquitination in vitro as well as Rsp5p binding and Mga2p polyubiquitination in cells. However, the Mga2pLPKY mutant undergoes efficient proteasome-dependent processing. These experiments indicate that the LPKY motif of Spt23p is required for Rsp5p binding, Rsp5-induced ubiquitination, proteasome-dependent processing, and its OLE1 inducing function. They also suggest that the LPKY motif of Mga2p is required for Rsp5p binding and ubiquitination, and Rsp5p regulates Mga2p function by a mechanism that is independent of providing the partial degradation signal.

Regulated ubiquitin proteasome-dependent processing is an uncommon event where the proteasome promotes limited rather than complete degradation of substrates. The best studied examples of proteins that undergo this process are the NF-κB precursors p105 and p100. Limited proteasome-dependent degradation of p105 (encoded by NF-κB1) generates p50, whereas partial proteolysis of p100 (encoded by NF-κB2) gives rise to p52 (1–4). Similar to the complete degradation process, incomplete proteolysis of p105 and p100 by the proteasome requires ubiquitin modification, and both proteins are substrates of the SGDy[cY] E3 ligase (5–7). Proteasome-mediated degradation initiates at or within the carboxyl terminus of the proteins and terminates downstream of the Rel homology domain (1–4, 8, 9). The highly stable structures present within the sd1 and sd2 regions of the Rel homology domain in NFKB1 suppress destruction of amino-terminal sequences (8–10). The glycine-rich region located carboxyl-terminal to the Rel homology domain is also necessary for limited proteolysis (4, 11–13), although the precise role that the glycine-rich domain plays in this process remains unclear. After proteolysis, p50 and p52 dimerize with p65, forming the NF-κB transcriptional regulatory complex (reviewed in Ref. 14). This complex is maintained in a latent state in the cytoplasm via an interaction with inhibitor proteins termed IκBs. Nuclear mobilization of the NF-κB complex occurs by signal-induced ubiquitin-proteasome-dependent degradation of IκBs.

Two homologous Saccharomyces cerevisiae transcription factors, Spt23p and Mga2p, also undergo limited proteasome-dependent degradation, and interestingly, they share many structural domains with p105 and p100 (15). Spt23p and Mga2p harbor ankyrin repeats as well as a Rel homology domain (also termed a Ig-like/plexins/transcription factor (IPT)1 dimerization domain). One major difference between the yeast and NF-κB proteins is that Spt23p and Mga2p contain a carboxyl-terminal transmembrane domain and are expressed as 120-kDa ER-anchored proteins (termed p120) (15). These proteins form homodimers at the ER membrane, followed by proteasome-dependent processing of one of the monomers (16). The processing events generate Spt23p and Mga2p polypeptides that migrate at ~90 kDa on SDS-polyacrylamide gels (termed p90) (15). Interestingly, the processed products remain tethered to the ER membrane via an interaction with the unprocessed monomer, and release of the processed products from the ER also appears to be dependent on the ubiquitin-proteasome pathway (16, 17). For Spt23p, it has been shown that monoubiquitination of Spt23p90 provides the signal for its mobilization to the nucleus by the Cdc48pNpl4pUfd1p sequestration complex (16). In contrast, Mga2p90 release from the ER has been linked to Mga2p120 polyubiquitination and Cdc48pNpl4pUfd1p-mediated separation of polyubiquitinated Mga2p120 from unmodified Mga2p90 (17). This presumably leads to degradation of Mga2p120 by the proteasome and nuclear translocation of Mga2p90 (17). Nevertheless, once liberated from the membrane, p90 polypeptides of Spt23p and Mga2p migrate to the nucleus where they up-regulate the expression of the essential yeast gene OLE1 (encodes Δ9 fatty acid desaturase, an enzyme involved in the synthesis of oleic acid) (15, 17).

§ The abbreviations used are: IPT, Ig-like/plexins/transcription factor; ER, endoplasmic reticulum; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; HA, hemagglutinin.

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The Rsp5p ubiquitin ligase is required for the OLE1 inducing function of Spt23p and Mga2p. Similar to spt23Δmga2Δ cells, the proliferation deficiency of rsp5Δ cells is rescued (at least in part) by supplementation of growth medium with oleic acid or transformation with spt23 or mga2 alleles lacking the transmembrane domain (15, 18). Rsp5p is a member of the highly conserved Nedd4 family of ubiquitin ligases, and these proteins harbor a Ca2+/phospholipid-binding (C2) domain, multiple protein-interacting modules termed WW domains, and a homologous E6-AP carboxyl terminus domain (reviewed in Ref. 19). Interestingly, Rsp5p has also been implicated in inducing the complete degradation of numerous proteins, including plasma membrane-localized permeases and transporters (20). However, the Rsp5p recognition sequences on many of these proteins have yet to be defined, and it still remains possible that their differential turnover in rsp5 mutant cells is an indirect result of losing Rsp5p function.

Although Rsp5p is dispensable for Mga2p processing and may activate its function by promoting release of the processed product from the ER (17), the ligase is required for proteasome-dependent processing of Sp23p (15). Rsp5p-induced processing of Spt23p requires WW domain 3 and the homologous E6-AP carboxyl terminus domain but not the C2 domain of the ligase (15). Rsp5p has also been shown to interact with Spt23p and Mga2p in cells as determined by co-immunoprecipitation and yeast two-hybrid analyses, perhaps pointing to a direct association between Rsp5p and these membrane-bound transcription proteins have yet to be defined, and it still remains possible that their differential turnover in rsp5 mutant cells is an indirect result of losing Rsp5p function.

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### EXPERIMENTAL PROCEDURES

#### Yeast Strains, Plasmids, and Antibodies—Yeast techniques and manipulations were performed according to standard protocols. Preparation of oleic acid containing media and plates as well as genotypes of rps5Δ, spt23Δ, mga2Δ, and appropriate isogenic yeast strains have been described by us previously (17). The temperature-sensitive protosomes deficient cim3-1 strain (MATa ara1-32 leu2-3 his3Δ200 cim3-1) was a kind gift from Charles Mann (Service de Biochimie et de Geneetique Moleculaire, Gif-sur-Yvette, France). The yeast expression construct for Mga-tagged Mga2p that is under control of its native promoter, pYE26/Mga2HAp, was kindly provided by Stefan Jentsch (Max Planck Institute of Biochemistry, Martinsried, Germany). Plasmids pQE30-His-RSps5WW1/23, pYes-FLAGmga2, pYes-FLAGMga2HAp, pYes-FLAGMga2ΔSH, pYes-FLAGmga2Δipt, pYEpLac181-Δmga2Δ2 ipt, pYes-FLAGmga2Δipt, pYes-FLAGmga2Δipt, pYEpLac181-Δmga2Δ2 ipt, and pESC-FLAGSpt23HAp have been described previously (17, 21). spt23Δmga2Δ and mga2Δmga2Δ mutant constructs (i.e., pYes-FLAGmga2Δipt, pYEpLac181-Δmga2Δipt, and pESC-FLAGSpt23Δipt) have been described previously (17, 21). Yeast cell pellets were resuspended in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% DTT, 2 mM EDTA, 1% Triton X-100, and 1 mM PMSF) and stored at 4 °C. After clarification by centrifugation, supernatant was transferred to a new tube, and the protein concentration was determined by the Bradford assay (Bio-Rad). For Western blotting, 5–50 μg of protein extract was resolved on 6–9% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with indicated antibodies. For immunoprecipitations, 1 μg of protein lysates prepared in RIPA buffer were diluted in an equal volume of 50 mM Tris HCl, pH 8.0, 150 mM NaCl supplemented with protease inhibitors. The protein extracts were first preclariﬁed with protein G-Sepharose (Amersham Biosciences) and then incubated with appropriate antibodies for 2.5 h at 4 °C with gentle rocking. Protein G-Sepharose was added, and the incubations were continued for an additional 2 h. The beads were pelleted and washed three times with RIPA buffer, and proteins were eluted by boiling in 1× SDS-PAGE loading buffer.

**In Vitro Ubiquitination Assay**—FLAG-Spt23p and FLAG-Mga2p were translated in the presence of [35S]-methionine in a wheat germ extract coupled in vitro transcription/translation system (TNT; Promega). 15 μl of the translation reaction was used for each ubiquitination reaction. Ubiquitination reactions contained the following in a total volume of 120 μl: 10 mM Tris, pH 7.5, 20 mM NaCl, 125 μM DTT, 5 mM MgCl2, 5 mM ATP, 50 μg/ml ubiquitin (Sigma), 20 ng of purified human E1 ubiquitin-activating enzyme, and 10 ng of purified yeast Ubc1p. Purified Rsp5p (50 ng) or buffer control was added to initiate the reactions, and the reactions were incubated for 40 min at room temperature. The reactions were then placed on ice and diluted to a total volume of 250 μl with buffer containing 25 mM Tris, pH 7.5, and 50 mM NaCl. 1 μl of anti-FLAG monoclonal antibody was added along with protein G-agarose, and the reactions were rotated at 4 °C for 1 h. The agarose beads were collected and washed with buffer containing 25 mM Tris, pH 7.5, 50 mM NaCl, and SDS-PAGE loading buffer was added to the washed beads. The beads were heated at 90 °C for 5 min, and the supernatants were loaded onto 10% SDS-polyacrylamide gels. The dried gels were exposed to film, and the proteins were detected by autoradiography.

**Northern Blotting**—RNA isolations and Northern blotting was performed as described previously (21) using 3P-radioabeled OLE1 or U2 cDNA (used as a loading control) probes.

### RESULTS

**The LPKY Motif of Spt23p Is Required for Rsp5p Binding in Vitro and in Vivo**—It has previously been documented that Rsp5p interacts with Spt23p via the WW domains of the ligase (15, 21). The WW domains of Rsp5p have been classified as Group I WW domains based on their direct binding preference for proline-containing sequences (22). Typically, Group I WW domains bind PPXY sequences, although it has been shown that the first proline position can be replaced by other amino acids such as serine or leucine (22). There is only one putative Group I WW domain-binding site located within Spt23p and this imperfect site (LPKY) is present carboxyl-terminal to the processing termination site of Spt23p (Fig. 1A). To determine whether this motif is required for Rsp5p binding, we performed in vitro association assays with the following: (i) polypeptide containing only the WW domains of the protein and (ii) translated full-length Spt23p or a Spt23p mutant lacking these four amino acids. As shown in Fig. 1B, we detected an interaction between the WW domains of Rsp5p and full-length Spt23p. However, only background binding was observed between the WW domain containing Rsp5p polypeptide and Spt23pΔLPKY, indicating that the LPKY motif mediates an interaction with the substrate binding domain of the ligase. To determine whether elimination of the LPKY motif abrogates an interaction between Rsp5p and Spt23p in cells, we co-expressed...
epitope tagged versions of Rsp5p and Spt23p (either full-length or the ΔLPKY mutant). Immunoprecipitations were performed with an antibody recognizing the amino-terminal FLAG tag on Spt23. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with an antibody recognizing amino-terminal HA-tagged Rsp5p. Fig. 1C shows the presence of Rsp5p in immunoprecipitations derived from cells expressing Rsp5p and Spt23p. Rsp5p was not detected in immunoprecipitations from cells extracts harboring Rsp5p and Spt23pΔLPKY or in any of the control immunoprecipitations. These results show that the LPKY motif of Spt23p is required for Rsp5p binding in vitro and in yeast, and considering the nature of the domains required for an interaction (i.e., type I WW domains and the LPKY motif), we conclude that the Rsp5p-Spt23p interaction is direct.

Deletion of the LPKY Motif Abrogates Rsp5p-induced Spt23p Ubiquitination in Vitro and Ubiquitination in Cells—Our next aim was to determine whether deletion of the LPKY motif abrogates Rsp5p-dependent Spt23p ubiquitination in vitro. In vitro ubiquitin conjugation reactions with Spt23p and Spt23pΔLPKY (translated in wheat germ extracts) were carried out in the presence of purified E1 and E2 enzymes in either the presence or the absence of recombinant Rsp5p. A recombinant catalytically inactive Rsp5p mutant harboring an alanine substitution at the carboxyl-terminal cysteine residue was also included as a control for this experiment. As shown in Fig. 2A, a marked increase in the amount of high molecular weight conjugates and a corresponding decrease in the level of unmodified Spt23p120 was observed in reactions containing purified Rsp5p. This Rsp5p-induced modification was not observed in reactions containing Spt23pΔLPKY or in control reactions with Spt23p and the ligase-deficient Rsp5p mutant. These results provide strong evidence for a direct physical and functional interaction between Spt23p and Rsp5p and suggest that this association is mediated by a single imperfect group I WW domain-binding motif that is located within the carboxyl terminus of the protein.

To ascertain that deletion of the WW domain-binding site affects Spt23p ubiquitination in cells, yeast were transformed with SPT23 or spt23Δlpky expression constructs. Spt23p proteins were immunopurified from cell extracts using an antibody recognizing a carboxyl-terminal HA epitope tag (this will be present only within unprocessed Spt23p120), resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were then probed with anti-ubiquitin or anti-HA antibodies. As shown in Fig. 2B (top panel), Spt23pΔLPKY is poorly ubiquitinated in vivo when compared with full-length Spt23p. Low amounts of ubiquitinated Spt23pΔLPKY could not be explained by decreased expression or inefficient immunoprecipitation because equivalent amounts of total Spt23p120 and Spt23p120ΔLPKY were present in the immunoprecipitations (Fig. 2B, bottom panel). These results suggest that deletion of the Rsp5p-binding LPKY motif abrogates Spt23p ubiquitination in cells and is consistent with the idea that this domain mediates a functional interaction with the ligase in vivo.

Deletion of the LPKY Motif Abrogates Spt23p Processing and Its OLE1 Inducing Function—To determine whether the identified Rsp5p-binding site is required for Spt23p processing, spt23Δipt cells were transformed with SPT23, spt23Δlpky or spt23Δipt expression constructs. The spt23Δipt construct was
included as a control for this experiment because previous studies have documented a requirement for the IPT dimerization domain in Spt23p processing (16). As shown in Fig. 3A, we could not detect Spt23p90 in cells harboring the IPT domain mutant, whereas only a very small amount of the processed product was detected in cells expressing Spt23p/H9004LPKY. Considering the reduced amount of Spt23p90 present in cells harboring spt23/H9004lpky, we reasoned that the encoded mutant is deficient at inducing target gene expression. To test this, we measured the amount of OLE1 transcripts (a Spt23p target gene) in cells expressing Spt23p, Spt23p/H9004LPKY, and Spt23p/H9004IPT. Fig. 3B shows dramatically lower amounts of OLE1 RNA in cells expressing Spt23p/H9004LPKY or Spt23p/H9004IPT when compared with cells expressing full-length Spt23p. These results suggest that the LPKY motif is required for proteasome-dependent processing and generation of its transcriptionally active form.

Elimination of the LPKY Motif on Mga2p Abrogates Rsp5p Binding and Ubiquitination—As stated in the introduction, Mga2p is also an ER localized transcription factor and is highly homologous to Spt23p in both structure and function (the proteins share 34% identity and 50% similarity at the amino acid level). Both proteins harbor the same domain organization, and they play overlapping roles in inducing OLE1 gene expression (18). It should be noted however that differences in their regulation and activity have been documented, suggesting that these proteins possess nonoverlapping functions (15, 17, 23). Interestingly, the LPKY motif and surrounding sequences of Spt23p (i.e. starting at amino acid 934, RINDDLPKYEDL) are highly similar in Mga2p (starting at amino acid 962, RLNDLPKYEDL). To determine whether deletion of the LPKY motif of Mga2p abrogates Rsp5p binding and Rsp5p-dependent ubiquitination, we performed the in vitro experiments described in Figs. 1 and 2 but for Mga2p. Similar to Spt23p, we found that deletion of the LPKY site of Mga2p abolished Rsp5p binding (Fig. 4A) and Rsp5p-dependent Mga2p ubiquitination in vitro (Fig. 4B).

To verify that the LPKY motif of Mga2p is required for Rsp5p binding in cells, we performed co-immunoprecipitation experiments. Although deletion of this motif affects Rsp5p binding, we noticed much less Rsp5p that co-immunoprecipitates with Mga2p when compared with Spt23p (Fig. 5A). Considering that
the cells used for this assay contain comparable amounts of input proteins and that equal amounts of Spt23p and Mga2p were present in the immuno-purified complexes (Fig. 5A), it is unlikely that low amounts of Rsp5p in the Mga2p immunopre-

cipitations are due to differential expression of proteins or unequal immunoprecipitation. The weak binding detected between Rsp5p and Mga2p promoted us to test whether deletion of the LPKY motif affects Mga2p ubiquitination in cells. Inter-
To determine whether deletion of the LPKY motif affects Mga2p processing, we expressed Mga2p or Mga2pLPKY in indicated galactose-inducible Mga2p expression constructs or plasmids containing MGA2 or indicated mga2 mutants that are under the control of the native MGA2 promoter. The antibodies used for these blots recognize the amino-terminal tag on the proteins and thus will detect the unprocessed p120 and processed p90 polypeptides. It is noted that Mga2pLPKY is underexpressed when compared with Mga2p when both proteins are placed under the control of the native MGA2 protein. Thus, lane 2 of the gel presented in the bottom panel contains 50 μg of protein lysates, whereas those in lanes 1 and 3 contain 5 μg. B, rsp5Δ cells containing galactose-inducible MGA2 or mga2Δlpky (derived from two independent colonies each) were grown in glucose medium containing oleic acid and then placed in galactose medium supplemented with oleic acid. The extracts were prepared from harvested cells, and Western blotting was performed with an antibody recognizing the amino-terminal epitope tag. C, proteasome-deficient yeast strain cim3-1 and control strain (CIM3) were transformed with galactose-inducible MGA2 or mga2Δlpky. The cells were grown in glucose medium at 25 °C and then placed in galactose medium and incubated at 37 °C (nonpermissive temperature for cim3-1 cells). The extracts were prepared from harvested cells, and Western blotting was performed with an antibody recognizing the amino-terminal epitope tag.

Fig. 6. Mga2pΔ120LPKY undergoes proteasome-dependent processing via an Rsp5p-independent mechanism. A, Western blotting was performed using extracts prepared from mga2Δ cells harboring the indicated galactose-inducible Mga2p expression constructs or plasmids containing MGA2 or indicated mga2 mutants that are under the control of the native MGA2 promoter. The antibodies used for these blots recognize the amino-terminal tag on the proteins and thus will detect the unprocessed p120 and processed p90 polypeptides. It is noted that Mga2pLPKY is underexpressed when compared with Mga2p when both proteins are placed under the control of the native MGA2 protein. Thus, lane 2 of the gel presented in the bottom panel contains 50 μg of protein lysates, whereas those in lanes 1 and 3 contain 5 μg. B, rsp5Δ cells containing galactose-inducible MGA2 or mga2Δlpky (derived from two independent colonies each) were grown in glucose medium containing oleic acid and then placed in galactose medium supplemented with oleic acid. The extracts were prepared from harvested cells, and Western blotting was performed with an antibody recognizing the amino-terminal epitope tag. C, proteasome-deficient yeast strain cim3-1 and control strain (CIM3) were transformed with galactose-inducible MGA2 or mga2Δlpky. The cells were grown in glucose medium at 25 °C and then placed in galactose medium and incubated at 37 °C (nonpermissive temperature for cim3-1 cells). The extracts were prepared from harvested cells, and Western blotting was performed with an antibody recognizing the amino-terminal epitope tag.

Interestingly, we noticed a dramatic decrease in the amount of ubiquitinated protein in yeast expressing Mga2pΔLPKY when compared with Mga2p (Fig. 5B). Reduced ubiquitination of the LPKY mutant is unlikely because of loss of a key ubiquitin conjugation site because substitution of the lysine present within this motif to an arginine does not negatively affect Mga2p ubiquitination in cells.2 We conclude from these studies that although Rsp5p does not associate as well with Mga2p when compared with Spt2p under the conditions employed here, the LPKY motif is required for an Rsp5p interaction and Rsp5p-dependent Mga2p ubiquitination.

Mga2pΔ120LPKY Undergoes Proteasome-dependent Processing via an Rsp5p-independent Mechanism—To determine whether deletion of the LPKY motif affects Mga2p processing, we expressed Mga2p or Mga2pΔLPKY in mga2Δ cells and measured the relative amount of Mga2p120 and Mga2p90. Similar to Spt2p3, the IPT domain of Mga2p has been shown to be required for proteasome-dependent processing, and thus cells harboring mga2Δipt were included as a control for these studies. As shown in Fig. 6A (top panel), efficient production of Mga2p90 was detected in cells expressing galactose-inducible Mga2pΔLPKY, whereas no Mga2p90 was detected in cells expressing the Mga2pΔIPT mutant. Similar results were obtained using Mga2p expression constructs that are under control of the native MGA2 promoter (Fig. 6A, bottom panel). These data indicate that processing of Mga2pΔLPKY is mediated by an Rsp5p-independent mechanism. To confirm this, we assessed processing of Mga2pΔLPKY in rsp5Δ cells and cells containing RSP5 (both cells were grown in oleic acid containing media). Fig. 6B shows no obvious perturbation of Mga2p or Mga2pΔLPKY processing in rsp5Δ cells. Processing of Mga2p and Mga2ΔLPKY was, however, severely affected in the temperature-sensitive proteasome-deficient cim3-1 (24) strain (Fig. 6C), suggesting that Mga2p90 generation is dependent on the proteasome but not on the Rsp5p ubiquitin ligase.

DISCUSSION

Previous cell-based studies have implicated the WW domain-containing Rsp5p as an E3 ubiquitin ligase for Spt23p and as an inducer of the proteasome-dependent processing signal (15). Work presented here extends these studies by defining an imperfect Group I WW domain binding site (i.e. LPKY) on Spt23p that is required for Rsp5p binding and Rsp5p-dependent ubiquitination in vitro. In addition, we show here that deletion of the LPKY site abrogates Spt23p ubiquitination, Spt23p90 generation, and Spt23p-induced OLE1 gene expression in cells. These results point to a direct interaction between Rsp5p and Spt23p and suggest that the LPKY motif mediates Rsp5p binding and Rsp5p-induced Spt23p ubiquitination in vitro. They also suggest that this interaction is required for limited proteasome-dependent degradation of Spt23p120 and generation of the transcriptionally active Spt23p90. It is interesting to note that the LPKY motif is located within the carboxyl terminus of Spt23p and is not present within the p90 form of Spt23p. Although it remains unclear whether ubiquitin conjugation occurs via lysines that are also localized in this region, the location of the Rsp5p interaction motif may be important for directing proteasome recruitment to the carboxyl-terminal region of the protein where the partial degradation process initiates (15).

As stated previously, the data presented in this study points to a direct physical and functional relationship between Rsp5p and Spt23p. However, we still do not know the nature of the Rsp5p-induced Spt23p processing signal. Although Rsp5p induces Spt23p120 polyubiquitination in vitro, it remains unclear whether this activity promotes proteasome-dependent processing, and it is possible that Rsp5p-dependent monoubiquitination and not polyubiquitination serves as the processing signal. Nevertheless, the findings presented here indicate that straightforward in vitro assays will be successful for identifying ubiquitin requirement for the limited degradation signal on Spt23p. Although Rsp5p is dispensable for Mga2p processing, the data presented here suggest that the proteasome mediates this event. Processing of both Mga2p and the Rsp5p-binding deficient mutant is suppressed in a proteasome-deficient yeast strain. Moreover, we have found that proteasome inhibitors suppress limited degradation of both Spt23p and Mga2p in a crude rabbit reticulocyte system.3 Although Mga2p processing is presumed to require ubiquitination, we still do not have hard evidence in hand to support this claim. In fact, we have found that it is hard to detect Mga2pΔLPKY ubiquitin conjugates in cells or reticulocytes, even though this mutant undergoes efficient proteasome-dependent processing in both systems. It is clear that more biochemically oriented approaches are needed to determine whether ubiquitination is required for proteasome-dependent processing of Mga2p and, if so, the identity of ligases that provide this signal.

The role that the Rsp5p-binding site plays in Mga2p function

2 N. Shcherbik and D. S. Haines, unpublished data.

3 S. Battyacharya, N. Shcherbik and D. S. Haines, unpublished data.
remains to be defined. It is clear that elimination of the LPKY motif negatively affects Rsp5p binding and Rsp5p-induced ubiquitination in vitro and in vivo. However, this mutant undergoes efficient processing in cells. These results are consistent with our past studies suggesting that proteasome-dependent Mga2p processing occurs via an Rsp5p-independent manner (17). Considering our past findings as well as the genetic data published by others (15, 18), it is likely that Rsp5p activates Mga2p function by a mechanism that is independent of promoting proteasome-dependent processing. We previously suggested that Rsp5p promotes liberation of transcriptionally active Mga2p90 by inducing polyubiquitination and degradation of the interacting Mga2p120 membrane-bound anchor (17). The Rsp5p binding and ubiquitination data presented here with the Mga2pLPKY mutant is consistent with this model and we have initiated experiments testing whether Mga2p90 is sequestered at the ER membrane in cells harboring mga2Δlpky and whether these cells express lower amounts of OLE1 transcripts. We have found slightly lower amounts of OLE1 transcripts and nuclearily localized Mga2p90 in cells harboring the LPKY deletion mutant using the galactose-inducible expression system. However, when evaluating these parameters under conditions where expression of MGA2 and mga2Δlpky are under the control of the native MGA2 promoter, we have noticed that Mga2pLPKY is poorly expressed in these cells (this appears to be at the plasmid copy level), making the Mga2p90 localization and OLE1 expression studies very difficult to interpret. We are currently establishing an in vitro mobilization assay that will hopefully allow us to more precisely define the role of Rsp5p in Mga2p90 release. It is, however, possible that Rsp5p regulates Mga2p function or expression by a mechanism that is independent of promoting processing or release of the processed product. It is also conceivable that Rsp5p plays a predominant role in Spt23p activation but only a minor role in Mga2p activation. If so, the growth deficiencies of rps5Δ cells could be a combined affect of a significant, but not necessarily complete, suppression of OLE1 expression and loss of other important Rsp5p-regulated pathways (25–31).

We were somewhat surprised to find that it is much easier to co-immunoprecipitate Rsp5p with Spt23p when compared with Mga2p under very similar experimental conditions. Considering that the LPKY motif appears to be the only Rsp5p-binding site present within the proteins, it is tempting to speculate that an interaction between Rsp5p and Mga2p is constitutively suppressed. If this is indeed the case and Rsp5p promotes Mga2p90 release from the ER by inducing polyubiquitination and degradation of the Mga2p120 membrane-bound anchor, it is possible that the block is removed under conditions where Mga2p90 activity is desired within the cell. Differential modulation of Rsp5p binding to these membrane-bound transcription factors could be an important regulatory mechanism for separating out the transcriptional regulatory functions of these proteins and/or providing a more precise control of unsaturated fatty acids levels within the cell.

The significance of localization of Spt23p and Mga2p at the ER membrane remains unclear. Because these proteins regulate the expression of a gene (i.e. OLE1) involved in the synthesis of unsaturated fatty acids (18), it is thought that their localization is relating to membrane-associated events that are tied to changes in fatty acid pools and membrane fluidity. Whether these signaling pathways directly regulate ligase-mediated processing of Mga2p or Spt23p remains to be determined. It is possible that ligase-dependent ubiquitination of the membrane-bound proteins requires membrane initiated signals that promote mobilization of ligases to the ER. Alternatively, it may be dependent on membrane-associated events regulating ligase binding to the substrate. On even a more basic level, it remains unclear whether Rsp5p-dependent ubiquitination of Spt23p or Mga2p is dependent on cooperating ER-localized E2 enzymes or recruitment of an Rsp5p-E2 complex to the ER. Future research in these areas will improve our basic understanding of how the ubiquitin-proteasome pathway impinges on the expression and activity of membrane-localized proteins in eukaryotic cells.

REFERENCES

1. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773–785
2. Chen, A., Whiteside, S., Israel, A., Stanczowski, I., Schwartz, A. L., and Ciechanover, A. (1995) J. Biol. Chem. 270, 21707–21714
3. Betts, J. C., and Nabel, G. J. (1996) Mol. Cell. Biol. 16, 6365–6371
4. Heusch, M., Lin, L., Gelezuiunas, R., and Greene, W. C. (1999) Oncogene 18, 6201–6208
5. Heissmeyer, V., Krappmann, D., Hatada, E. N., and Scheiderer, C. (2001) Mol. Cell. Biol. 21, 1024–1035
6. Orian, A., Gonen, H., Berovich, B., Fajerman, I., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Ciechanover, A. (2000) EMBO J. 19, 2580–2591
7. Fang, A., and Sun, S. C. (2002) J. Biol. Chem. 277, 22111–22114
8. Lin, L., and Kobayashi, M. (2003) J. Biol. Chem. 278, 31479–31485
9. Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., and Matuschek, A. (2001) Mol. Cell 7, 627–637
10. Lin, L., DeMartino, G. N., and Greene, W. C. (2000) EMBO J. 19, 4712–4722
11. Lin, L., and Ghosh, S. (1996) Mol. Cell. Biol. 16, 2248–2254
12. Orian, A., Schwartz, A. L., Israel, A., Whiteside, S., Kahana, C., and Ciechanover, A. (1999) Mol. Cell. Biol. 19, 3664–3673
13. Ciechanover, A., Gonen, H., Berovich, B., Cohen, S., Fajerman, I., Israel, A., Mercurio, F., Kahana, C., Schwartz, A. L., Iwai, K., and Orian, A. (2001) Biochimie (Paris) 83, 341–349
14. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
15. Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H. D., and Jentsch, S. (2000) Cell 102, 577–586
16. Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (2001) Cell 107, 667–677
17. Scherberik, N., Zoladek, T., Nickels, T. J., and Haines, D. S. (2003) Curr. Biol. 13, 1227–1233
18. Zhang, S., Skalsky, Y., and Garfinkle, D. J. (1999) Genetics 151, 473–483
19. Ingraham, R. J., Gish, G., and Pawson, T. (2004) Oncogene 23, 1972–1984
20. Rotin, D., Staub, O., Haguenauer-Tsapis, R. (2000) J. Membr. Biol. 176, 1–17
21. Scherberik, N., Kumar, S., and Haines, D. S. (2002) J. Cell Sci. 115, 1041–1048
22. Chang, A., Cheang, S., Espanel, X., and Sudol, M. (2000) J. Biol. Chem. 275, 20562–20571
23. Jiang, Y., Vanconcelles, M. J., Wretzel, S., Light, A., Martin, C. E., and Goldberg, M. A. (2001) Mol. Cell. Biol. 18, 6161–6169
24. Ghislain, M., Udvardy, A., and Mann C. (1993) Nature 366, 358–362
25. Neumann, S., Petfalski, E., Brugger, B., Grosshans, H., Wieland, P., Tollervey, D., and Hure, E. (2003) EMBO Rep. 4, 1156–1162
26. Rodriguez, M. S., Gwizdek, C., Haguenauer-Tsapis, R., and Dargemont, C. (2003) Traffic 4, 566–575
27. Kaminska, J., Gajewska, B., Hopper, A. K., and Zoladek T. (2002) Mol. Cell. Biol. 22, 6846–6848
28. Haynes, C. M., Caldwell, S., Cooper, A. A. (2002) J. Cell Biol. 158, 91–101
29. Beaudenon, S. I., Husanci, M. R., Wang, G., McDonnell, D. P., and Hubergetse, J. M. (1999) Mol. Cell. Biol. 19, 6972–6979
30. Fisk, H. A., and Yaffe MP. (1999) J. Cell Biol. 145, 1199–1208
31. Dunn, R., and Hicke, L. (2001) J. Biol. Chem. 276, 25974–25981