Identification of Pep4p as the Protease Responsible for Formation of the SAGA-related SLIK Protein Complex

Received for publication, January 28, 2010, and in revised form, May 12, 2010 Published, JBC Papers in Press, May 24, 2010, DOI 10.1074/jbc.M110.108787

Gianpiero Spedale, Nikolai Mischerikow, Albert J. R. Heck, H. T. Marc Timmers, and W. W. M. Pim Pijnappel

From the Department of Physiological Chemistry, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands Proteomics Centre, Padualaan 8, 3584 CH Utrecht, the Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Centre for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Padualaan 8, 3584 CH Utrecht, and the Centre for Biomedical Genetics, Padualaan 8, 3584 CH Utrecht, The Netherlands

The Saccharomyces cerevisiae Spt-Ada-Gcn5 acetyltransferase (SAGA) protein complex is a coactivator for transcription by RNA polymerase II and has various activities, including acetylation and deubiquitination of histones and recruitment of TATA-binding protein to promoters. The Spt7p subunit is subject to proteolytic cleavage at its C terminus resulting in removal of the Spt8p-binding domain and generation of the SAGA-related SALSA/SAGA-like (SLIK) protein complex. Here, we report identification of the protease responsible for this cleavage. Screening of a protease knock-out collection revealed PEP4 to be required for cleavage of Spt7p within SAGA in vitro. Endogenous formation of truncated Spt7p was abolished in cells lacking PEP4. Purified Pep4p but not catalytic dead mutant Pep4p or unrelated Prc1p protease specifically cleaved Spt7p within SAGA into SLIK-related Spt7p. Interestingly, SAGA lacking Spt8p was more sensitive to Pep4p-mediated truncation of Spt7p, suggesting that Spt8p counteracted its own release from SAGA. Strains mimicking constitutive SLIK formation showed increased resistance to rapamycin treatment, suggesting a role for SLIK in regulating cellular responses to nutrient stress.

Transcription initiation by RNA polymerase II is a dynamic process that is regulated by the interplay of a large number of factors. The class of coactivators orchestrates preinitiation complex formation by recruiting nucleosome remodelers, basal transcription factors, and histone modifiers. The evolutionary highly conserved Spt-Ada-Gcn5 acetyltransferase (SAGA) complex is a 1.8-MDa coactivator crucial for preinitiation complex assembly and transcriptional regulation of 10% of the genes in Saccharomyces cerevisiae (1–5). SAGA contains ~21 different subunits organized in a multifunctional submodular architecture comprising a structural core, a Tra1p subunit, a histone acetyltransferase module, a deubiquitination module, and the Spt3p/Spt8p/TBP module (6–8). It is well established that the SAGA coactivator complex is recruited to upstream activating sequences by interaction of its Tra1p subunit with transcriptional activators (9, 10). On chromatin, SAGA acetylates the tails of histone H3 and H2B via its Gcn5p subunit, and it deubiquitinates histone H2B at lysine 123 via its Ubp8p subunit (11–13). SAGA is also involved in recruitment of basal transcription factors like TBP to the preinitiation complex via the Spt3p/Spt8p module (14, 15). Furthermore, SAGA interacts with other protein complexes, extending its functions to transcription elongation, mRNA export (16–18), and nucleosome remodeling (19, 20). SAGA in higher eukaryotes operates in telomere maintenance (21) and development (22). The Spt3p and Spt8p subunits of SAGA interact with TBP, modulating both positive and negative effects on gene expression (23). It is not clear which molecular mechanism governs the Spt8p–Spt3p–TBP interaction within SAGA, but several genetic and biochemical studies have described different aspects of this interplay. For instance, it has been shown that Spt8p binds directly TBP (8) and can compete with TATA box DNA for TBP binding (24). Furthermore, genetic evidence suggests that both Spt3p and Spt8p interact with TBP (25). This is indicated by the identification of mutations in TBP that are able to suppress SPT3 mutations in an allele-specific manner. The same TBP mutations also bypass SPT8-null mutations.

The identification of the SALSA/SAGA-like (SLIK) complex, here referred to as SLIK, has been previously reported in yeast (26–28). SLIK subunit composition is identical to that of SAGA except that in SLIK the Spt7p subunit is C-terminally truncated by proteolytic cleavage, leading to release of the Spt8p subunit. Furthermore, Rtg2p has been reported to be uniquely present in SLIK but not in SAGA (26), linking SLIK to the retrograde response pathway. Rtg2p has been shown to be required for the integrity of SLIK formation, and yeast deleted for either RTG2 or SPT8 displayed differential sensitivity to growth on alternative carbon sources like acetate and glycerol (26). It has also been shown that SLIK is actively involved in transcription of the HIS3 gene, thereby counteracting the inhibiting action of SAGA (27). Other analyses indicate redundancy of SLIK with SAGA (29). For instance, it has been shown that the Sgf73p subunit serves as anchor for the deubiquitination module in both SAGA and SLIK complexes (30). So far, the molecular function of SLIK has not been established with certainty.

This work was financed by Netherlands Genomics Initiative Horizon Program Grant 93516050 and by the Netherlands Proteomics Centre.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3, Tables 1–3, and additional references.

1. To whom correspondence should be addressed: Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. E-mail: w.w.m.pijnappel@umcutrecht.nl.

2. The abbreviations used are: SAGA, Spt-Ada-Gcn5 acetyltransferase; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; SLIK, SALSA/SAGA-like; WCE, whole cell extract; WT, wild type; TAP, tandem affinity purification; TBP, TATA-binding protein; TEV, tobacco etch virus.

This is an Open Access article under the CC BY license.

JULY 23, 2010•VOLUME 285•NUMBER 30
Pep4p Generates SLIK

possible function can be inferred from its distinct complex composition. In SLIK, the balance between the TBP-binding proteins Spt3p and Spt8p is broken because Spt8p is lost. This might affect the TBP delivery function of SAGA globally or specifically at promoters. We previously showed that cleavage of Spt7p occurs between positions 1141 and 1142 (31), which map in a cleavage domain described previously by Wu and Winston (29). This cleavage is essential for formation of the SLIK protein complex, but the protease responsible for this process has not been identified so far.

Site-specific proteolysis is involved in the regulation of gene expression by cleavage of transcription factors. Regulated intramembrane proteolysis, for instance, regulates the activation of SREBP, Notch, and ATF6 transcriptional factors (32). The cleavage of host cell factor regulates cell proliferation (33) whereas protein truncation of transcription factor IIA (34) or mixed-linkage leukemia protein (35) by Taspase1 protease serves to fine tune transcription regulation of a subset of genes during differentiation and development. Interestingly, it has also been shown that proteolytic cleavage of histone tails acts in chromatin regulation. In yeast, the histone H3 tail clipping after Ala^21 regulates gene expression (36), and in mouse cathepsin L cleaves H3 tails at position 21–22 and 27–28 during embryonic stem cell differentiation (37). The identification of proteases responsible for such functional protein truncation has been crucial for a better understanding of the biological processes listed above. Here, we identify Pep4p as the protease responsible for the cleavage of Spt7p and for the formation of the SLIK protein complex. Furthermore, we found that the SAGA-specific subunit Spt8p negatively regulates the Pep4p-mediated cleavage of Spt7p. Pep4p-mediated SLIK formation caused increased resistance to rapamycin treatment, suggesting a role for SLIK in the response to nutrient starvation.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Growth Conditions—Yeast strains used in this study were FY or W303-1B derivatives and are listed in supplemental Table 1. Gene tandem affinity purification (TAP) tagging and generation of null mutations were performed by standard methods as described previously (31). Protease expressing strains were constructed in W303-1B background. Plasmids for protease expression are listed in supplemental Table 3. Strains were selected on G418 plates and grown until saturation in standard liquid YPD.

Spot Assay and Rapamycin Treatment—Exponential phase growing strains were harvested and spotted in a dilution series from 10^6 to 10^4 cells/ml on synthetic complete medium (SC) supplemented with glucose (Glu) and containing rapamycin at a final concentration of 10, 20, or 30 nM. The plates were incubated at 30 °C for the following times: SC/Glu (2 days), 10 nM rapamycin (3 days), 20 nM rapamycin, and 30 nM rapamycin (4 days). Strains used were: WT (FY2031 (29)), SLIK-only (FY2032 (29)), Δspt8 (YPG160), Δpep4 (YPG161).

Yeast Extracts and Affinity Purifications—Yeast extracts for in vitro cleavage assay were prepared as described previously (31). Briefly, cell pellets were washed once with water and snap-frozen in dry ice. Pellets were thawed, resuspended in 2 ml of buffer E (10 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 10% glycerol) without protease inhibitors and transferred to 2-ml screw cap Eppendorf tubes with 1 ml of glass beads (BioSpec). Proteins were extracted by eight cycles (30-s power/2-min pause) in a minibeadbeater (BioSpec). Extracts were cleared by centrifugation at 14,000 rpm, and supernatants were aliquoted and snap-frozen for further analysis. Yeast extracts for in vivo Spt7p truncation analysis were prepared as above, but using buffer E with protease inhibitor mixture for yeast extracts (Sigma). SAGA and SLIK complexes, SAGA-only complex, and SAGA complex without Spt8p were purified, respectively, from WCEs of Coy142, FY2031, or YPG160 by TAP purification as described previously (31) with the following modifications: for the first binding step, 200 μl of streptavidin M-280 Dynabeads (Invitrogen) were washed with 3 ml of buffer E and resuspended in 180 μl of the same buffer. Beads were coated with 20 μl of biotinylated IgG (Bethyl) by incubation at 37 °C for 30 min with shaking at 1000 rpm. The beads were washed with 3 ml of buffer E and incubated with 10 ml of yeast cell extract for 2 h. Beads were collected, washed with 3 ml of buffer E, 1 ml of cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.1% Tween 20, and 1 mM dithiothreitol) and resuspended in 1 ml of cleavage buffer. The protein A moiety of the TAP tag was cleaved off with 10 μl of tobacco etch virus (TEV) protease (Invitrogen) for 2 h at 19 °C. The eluate was added to calmodulin-agarose beads (Stratagene) resuspended in 3 ml of calmodulin-binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl_2, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, and 10 mM β-mercaptoethanol) and incubated at 4 °C for 1 h. Beads were then washed with 30 ml of calmodulin-binding buffer, and proteins were eluted with 400 μl of elution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, and 10 mM β-mercaptoethanol). Eluates were aliquoted and stored at −80 °C or used directly for further analysis. Pep4p, Pep4p D294A, and Prc1p proteases C-terminally tagged with His_6-HA-prescision-protein A were affinity-purified from WCEs of the YGP151, YGP152, YGP153 strains, respectively. Cell pellets were resuspended in buffer E with 500 mM NaCl instead of 150 mM without protease inhibitors, and
RESULTS

Aspartic Protease Cleaves Spt7pSAGA into Spt7pSLIK—In search of the protease responsible for Spt7p cleavage and SLIK protein complex formation, we developed an in vitro cleavage assay in which Spt7p present in SAGA (Spt7pSAGA) is cleaved into Spt7p present in SLIK (Spt7pSLIK). As shown previously, SLIK together with the SAGA complex can be purified using a protein extraction performed as described previously (31). Protein A purification was carried out as described above. Beads were then collected and washed with 2 ml of prescission buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). Proteases were eluted with 5 μl/H9262 of PreScission protease (GE Healthcare) in 400 μl of prescission buffer at 10 °C for 4 h. 10% (v/v) glycerol was added to the eluate, and aliquots were stored at −80 °C for further analysis. Purifications were analyzed on NuPage 4–12% BisTris gradient gels with MOPS running buffer (Invitrogen) according to the manufacturers’ instructions. Subunits were identified by mass spectrometry (31). Molecular mass markers were obtained from New England Biolabs. Spt7p was detected using an HA antibody recognizing an N-terminally fused HA epitope. Inhibition of proteolysis was assayed by preincubation of the WCEs or purified proteases on ice for 30 min with the following protease inhibitors: 1 μg/ml pepstatin (Sigma), 1 mM AEBF (Sigma), 10 μM E64 (Sigma), or 1% (v/v) broad specificity protease inhibitor mixture P8250 (Sigma). Antibodies used were anti-HA antibody 3F10 (Sigma), anti-Spt7p antibody (a gift from F. Winston), anti-Spt8p antibody (a gift from J. Workman), anti-TAP antibody PAP (Sigma), and anti-tubulin (Immunologicals).

In Vitro Cleavage Assay, SDS-PAGE, and Immunoblot Analysis—The substrate for the in vitro cleavage assay was prepared as follows. FY2031 WCE was prepared as above to yield the SAGA preparations devoid of SLIK via purification of Spt7p through its C-terminal TAP tag. The in vitro cleavage assay included the following: 4 μl (or otherwise indicated) of yeast extract or purified protease, 10 μl of substrate in a total volume of 15 μl. Incubation was set at 30 min at 30 °C unless otherwise stated. The reaction was stopped by the addition of Laemmli buffer and incubation for 5 min at 95 °C. Reactions were run on 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Molecular mass markers were obtained from New England Biolabs. Spt7p was detected using an HA antibody recognizing an N-terminally fused HA epitope. Inhibition of proteolysis was assayed by preincubation of the WCEs or purified proteases on ice for 30 min with the following protease inhibitors: 1 μg/ml pepstatin (Sigma), 1 mM AEBF (Sigma), 10 μM E64 (Sigma), or 1% (v/v) broad specificity protease inhibitor mixture P8250 (Sigma). Antibodies used were anti-HA antibody 3F10 (Sigma), anti-Spt7p antibody (a gift from F. Winston), anti-Spt8p antibody (a gift from J. Workman), anti-TAP antibody PAP (Sigma), and anti-tubulin (Immunologicals).

FIGURE 1. Aspartic enzymatic activity cleaves Spt7pSAGA into Spt7pSLIK. A, purified SAGA and SLIK complex were stained with Coomassie. SPT20-C-TAP was used to purify both SAGA and SLIK (left lane). N-HA-SPT7-C-TAP was used to purify SAGA-only (right lane). Positions of full-length (Spt7pSAGA) and C-terminally truncated (Spt7pSLIK) forms of Spt7p as well as other SAGA/SLIK subunits are indicated. Identification of SAGA/SLIK subunits by mass spectrometry has been reported by us previously (31). B, activity present in yeast WCE cleaves Spt7pSAGA into Spt7pSLIK. Purified SAGA-only was incubated with yeast WCE for 30 min at 30 °C, and Spt7p processing was monitored by immunoblotting using an anti-HA antibody. Anti-tubulin antibody was used as a control for the amount of WCE added. FY2031 (N-HA-SPT7-C-TAP) WCE was used as positive control for in vivo generated Spt7pSLIK. FY2032 (29) immunoprecipitate (IP) was prepared by TAP purification and represents a genetic mimic of Spt7pSLIK. C, Spt7p-processing activity present in WCE belongs to the aspartic protease family. In vitro cleavage assay was as in B, but after inactivation of the WCE at 95 °C or with addition of protease inhibitors as indicated.
Pep4p Generates SLIK

FY2031 as positive control for the size of Spt7pSLIK. In this strain, Spt7pSLIK is produced from N-HA-Spt7p-C-TAP in vivo and can be detected by the anti-HA antibody (Fig. 1B, lane 5). As a second positive control we used a protein A-purified Spt7p in which the C-terminal part of Spt7p was deleted, mimicking Spt7pSLIK (29) (Fig. 1B, lane 4). To characterize the activity responsible for Spt7pSLIK formation further, we inactivated the WCE by 5 min at 95 °C, or we added protease inhibitors. Fig. 1C shows that both heat treatment and addition of the aspartic protease inhibitor pepstatin inhibited cleavage of Spt7p, whereas AESBF and E64 treatment had no effect. This indicates that an aspartic protease activity is responsible for the generation of Spt7pSLIK.

Knock-out Screen Reveals that Pep4p Protease Is Required for Cleavage of Spt7pSAGA into Spt7pSLIK—To identify the protease for Spt7p, we screened a yeast protease knock-out collection for Spt7p cleavage activity. The S. cerevisiae genome encodes a total of 121 proteases with a viable knock-out phenotype. WCEs of 89 knock-out strains (Open Biosystems) belonging to all protease classes were prepared and were tested using the in vitro cleavage assay described in Fig. 1B. Interestingly, 87 of 89 WCEs tested contained similarly high activity toward processing of Spt7pSAGA into Spt7pSLIK (supplemental Fig. S1B). Two WCEs, nos. 2 and 35, which lack Prb1p and Pep4p, respectively, showed moderately reduced (no. 2) or strongly reduced (no. 35) activity toward Spt7pSAGA processing. WCEs from these two candidates were retested for their cleavage activity on Spt7pSAGA as above, and the WCE total protein content was monitored by anti-tubulin immunoblot analysis. Consistent with the results from the screen, no proteolytic activity was detected in Δpep4 WCE, and a lower activity was present in the Δprb1 WCE compared with WT WCE (Fig. 2A). In yeast, the serine protease Prb1p activates Pep4p by cleavage of the N-terminal inhibitory domain. Pep4p can also cleave itself in the absence of Prb1p resulting in an active but less stable conformation (for review, see Ref. 38). Therefore, the absence of Prb1p leads to a reduction of Pep4p activity in yeast cells. We tested whether the low proteolytic activity detected in the Δprb1 WCE was related to reduced Pep4p protease activity. We employed the in vitro cleavage assay using Spt7pSAGA but now preincubated the Δprb1 WCE on ice with inhibitors for the three major classes of protease inhibitors classes as indicated (Fig. 2B). Inhibition of aspartic (pepstatin), but not serine (AESBF) and cysteine (E64) proteases reduces the cleavage of Spt7pSAGA (Fig. 2B). This is consistent with the hypothesis that the residual activity present in the Δprb1 WCE may be caused by autoactivation of the aspartic protease Pep4p.

We next tested whether Pep4p is required for Spt7p cleavage in vivo. To test this, we used an N-terminal TAP-tagged SPT7 strain in which Spt7pSAGA and Spt7pSLIK can be detected by anti-TAP immunoblot analysis. WT cells indeed contain both SAGA and SLIK forms of Spt7p (Fig. 2C). Deletion of PEP4 abolished the Spt7pSLIK formation whereas deletion of PRB1 or PRC1 had no effect on Spt7p cleavage (Fig. 2C). Taken together, these results indicate that Pep4p is required for cleavage of Spt7pSAGA into Spt7pSLIK both in vivo and in vitro.

Pep4p Protease Directly Cleaves Spt7p within the SAGA Complex—To investigate further the role of Pep4p in Spt7pSLIK formation, we expressed and purified tagged versions of Pep4p WT, a catalytically inactive Pep4p (mutation D294A) and the unrelated Prc1p protease from yeast overexpression strains (supplemental Fig. S2). As shown previously, two bands were present in the Pep4p purification due to autoactivation: the upper one is related to the proteolytic inactive form, and the lower one represents the active form. The Pep4p D294A catalytic dead mutant protease purification showed only the inactive form confirming the loss of proteolytic activity (38). These protease preparations were used in Spt7p cleavage assay, and immunoblot analysis revealed that incubation with the WT Pep4p protease but not with Pep4p D294A or Prc1p results in Spt7pSLIK formation (Fig. 3A). To confirm further that Pep4p was responsible for this activity we performed the assay as above but preincubated the protease with pepstatin for 30 min on ice. We observed that indeed the proteolytic activity of Pep4p on Spt7pSAGA was reduced in the presence of pepstatin (Fig. 3B).
Pep4p Generates SLIK

FIGURE 3. Purified Pep4p directly cleaves Spt7pSAGA to Spt7pSLIK. A, purified Pep4p cleaves Spt7pSAGA into Spt7pSLIK. Purified Pep4p along with control protease purifications (Pep4p D294A, Prc1p) were tested for in vitro cleavage of N-terminus of Spt7pSAGA for 30 min at 30 °C. Detection of cleavage was obtained using HA antibody. FY2032 IP was used as size control for the Spt7pSLIK generated. B, protease activity of Pep4p on Spt7pSAGA is pepstatin-sensitive. In vitro cleavage assay was carried out as in A but with a 30-min preincubation on ice with the aspartic protease inhibitor pepstatin. FY2032 IP was used as size control for the cleavage product. C, Pep4p specifically cleaves Spt7pSAGA within the SAGA complex. In vitro cleavage assay was performed as in A, and the reaction was analyzed on 4–12% acrylamide gel by Coomassie staining.

FIGURE 4. Spt8p interferes with Spt7pSLIK Formation. Pep4p protease activity is more efficient on SAGA-only complex lacking Spt8p (SAGA complex w/o Spt8p) compared with SAGA-only complex (SAGA complex). Purified complexes were incubated with purified Pep4p protease at 30 °C for 30 min, and truncation of Spt7pSAGA into Spt7pSLIK was detected by HA immunoblotting.

FIGURE 5. Pep4p-mediated SLIK formation is involved in rapamycin resistance. Rapamycin (rapa) sensitivity of strains deleted for PEP4 or mimicking constitutive presence of SLIK using FY2032 (SLIK only; lacking the C terminus of Spt7 as in SLIK (29)) or by deletion of SPT8. Cells were spotted in a dilution series from 10^6 to 10^3 cells/ml.

other subunits as well. We performed the in vitro cleavage assay using purified SAGA as described above (without pepstatin preincubation) but now used Coomassie staining to visualize all SAGA subunits (Fig. 3C). The migration of other SAGA subunits except Spt7p was not affected by incubation with Pep4p protease whereas Spt7pSAGA was processed specifically to Spt7pSLIK. This suggests that Pep4p specifically targets Spt7p within SAGA and does not cause a general proteolysis of SAGA subunits. Furthermore, the Coomassie staining analysis indicates that a substoichiometric amount of Pep4p is sufficient to cleave Spt7pSAGA. Taken together, these results demonstrate that Pep4p directly cleaves Spt7pSAGA into Spt7pSLIK with high efficiency and specificity.

Spt8p Interferes with Spt7pSLIK Formation—We showed previously that the cleavage at the C terminus of Spt7p occurs between the residues 1141 and 1142 or 1142 and 1143 (31). The cleavage site resides in the truncation domain flanked by the Spt8p-binding domain (29). This suggested the possibility that Spt8p binding to Spt7p may interfere with Spt7p cleavage. To test this we purified a SAGA-only complex via SPT7-C-TAP from a yeast strain with a deletion of SPT7 (SAGA complex without Spt8p) (supplemental Fig. S3). Deletion of SPT8 does not disrupt SAGA (29) as illustrated by the copurification of Gcn5p (supplemental Fig. S3). We incubated SAGA complex without Spt8p or SAGA complex with a limiting amount of purified Pep4p to detect differential sensitivities, and we found that the Spt7pSLIK formation was more efficient in the absence of Spt8p (Fig. 4). This suggests that Spt8p reduces Pep4p-induced cleavage of Spt7p, possibly by steric hindrance in which the accessibility of the cleavage site in Spt7p for Pep4p is blocked by the nearby binding of Spt8p.

Pep4p-mediated SLIK Formation Is involved in Rapamycin Resistance—Rapamycin is a widely used drug that is known to mimic nitrogen starvation by inhibiting the target of rapamycin (TOR) complex. Responses to rapamycin treatment are numerous and include large changes in gene expression programs such as activation of the nitrogen discrimination pathway and retrograde response pathway and repression of genes involved in cell growth and proliferation (39). Among the genes activated by rapamycin is also PEP4 (40). Yeast cells deleted for PEP4 show increased sensitivity to rapamycin treatment (Fig. 5) (40), indicating that Pep4p is required for growth under rapamycin conditions. Interestingly, yeast cells in which the forced presence of SLIK was mimicked, either by deletion of SPT8 or by deletion of the C terminus of SPT7 as in SLIK (SLIK-only), showed increased resistance to rapamycin treatment (Fig. 5). This suggests that SLIK formation by Pep4p is involved in the rapamycin response of yeast cells and confers resistance to this treatment. It is likely that SLIK functions as a coactivator to
regulate the changes in gene expression programs in response to nutrient starvation.

**DISCUSSION**

Here, we identify Pep4p as the protease responsible for Spt7p cleavage. Purified Pep4p specifically cleaves Spt7pSAGA into Spt7pSLIK within the SAGA complex in vitro, and genetic deletion of *PEP4* completely abolishes Spt7pSLIK formation in vivo. The Spt8p SAGA subunit reduces Spt7p cleavage and hence its release from the SAGA complex. Strains mimicking constitutive SLIK formation showed increased resistance to rapamycin treatment, suggesting a function for SLIK in the response to nutrient starvation.

The Pep4p protease belongs to the class of pepsin-like aspartic proteases (for review, see Ref. 38). It localizes predominantly to the vacuolar compartment, but translocation of this protease from the vacuole to the cytosol has been described. The active enzyme migrates from the vacuole in H$_2$O$_2$-triggered apoptotic cells (41). Translocation is associated with an increase in vacuolar permeability. This correlates with the degradation of nucleoporins resulting in an increase of nuclear pore complex permeability. Pep4p shares 40–60% identity with the human lysosomal proteases cathepsin D and cathepsin E. Cathepsin D can trigger lysosome-mediated apoptosis in mammalian cells (42). It migrates from the lysosome via an unknown mechanism and exerts its proteolytic function in a regulated manner (43). Interestingly, the cysteine protease cathepsin L also migrates from the lysosomal compartment to exert site-specific proteolytic cleavage. Upon translocalization to the nucleus, cathepsin L has been shown to be responsible for the processing of the transcription factor CDP/Cux (44) and for histone H3 clipping during embryonic stem cell differentiation (37). These results indicate that these vacuolar/lysosomal proteases do not only have degradative functions but that they are involved in regulated protein processing as well.

A previous study found that the cleavage of Spt7p does not require its incorporation into SAGA (45). Our findings indicate that cleavage of Spt7p can occur in the context of SAGA using an *in vitro* cleavage assay. Two possible mechanisms can be envisioned for cleavage of Spt7pSAGA into Spt7pSLIK and in turn for SLIK protein complex formation. First, Spt7p may be truncated in the cytosol prior to assembly into SLIK complex. Second, SLIK formation may be subsequent to the truncation of Spt7p within the SAGA complex, and this would most likely occur in the nucleus. In the latter case, truncation of Spt7p would lead to the release of Spt8p from the SAGA complex. Both genetic and biochemical evidence indicates that Spt8p interacts with TBP (24, 46). To investigate this point further, we used Spt8p as bait in tandem affinity purification and observed that substoichiometric amounts of SAGA were copurified, indicating that the majority of Spt8p in yeast cells is not in complex with SAGA (data not shown). This suggests that a pool of free Spt8p is present in yeast cells and that the binding to and release from its Spt7p partner within SAGA may be dynamically regulated. We showed that Spt8p interferes with Spt7p cleavage *in vitro* (Fig. 4). As a consequence, Spt8p may inhibit its own release from SAGA by reducing Pep4p-induced cleavage of Spt7p. This effect is likely to be sterical due to the close vicinity of the Spt8p-binding domain to the cleavage region in Spt7p (29). Truncation of Spt7p in *in vivo* may depend on a balance between cellular concentrations of Spt8p and Pep4p, which may be regulated by specific signals. Increased Pep4p levels upon nitrogen starvation or stress (40, 47, 48) may increase Spt7p cleavage and as a result Spt8p release. It is interesting to note that yeast strains deleted for the Ubp8p subunit display a reduced formation of SLIK, which suggests that Ubp8p can stimulate Pep4p-mediated cleavage of Spt7p (45).

Using rapamycin to mimic conditions of nitrogen starvation, we obtained evidence that Pep4p-mediated SLIK formation is important for the resistance to nitrogen starvation. Interestingly, deletion of several subunits shared between SAGA and SLIK has been shown to increase the sensitivity to rapamycin (49) and is in line with the idea that disruption of SLIK is involved in this phenotype. The deletions of *SPT8* or the C terminus of *SPT7* used here specifically mimic SLIK formation and allow us to address specifically the function of SLIK. Our results are consistent with the proposed function of SLIK in regulating genes involved in the retrograde response pathway upon nitrogen starvation (26). Rtg2p, a key transcription factor of the retrograde response pathway, has been reported to associate with SLIK (26). We hypothesize that increased Pep4p levels during nitrogen starvation induce Spt7p cleavage, Spt8p release from SAGA, and SLIK formation, which in turn regulates expression of genes important to respond properly to the changed nutritional status. The release of Spt8p is thought to change the TBP delivery function of SAGA. Future studies should determine the effect of Spt7p truncation by Pep4p on TBP recruitment and on the cellular responses to rapamycin.

**Acknowledgments**—We thank Fred Winston for strains and antibodies; Jerry Workman and Anthony Weil for antibodies; Frank Holstege, Tineke Lenstra, and Joris Benshop for plasmids and yeast strains; Nikolay Outchkourov and Radhika Warrier for helpful comments on the manuscript; and Folkert van Werven and the other Timmers laboratory members for discussion and suggestions.

**REFERENCES**

1. Bhaumik, S. R., and Green, M. R. (2001) *Genes Dev.* **15**, 1935–1945

2. Lee, T. I., Causton, H. C., Holstege, F. C., Shen, W. C., Hannett, N., Jennings, E. G., Winston, F., Green, M. R., and Young, R. A. (2000) *Nature* **405**, 701–704

3. Baker, S. P., and Grant, P. A. (2007) *Oncogene* **26**, 5329–5340

4. Lee, K. K., and Workman, J. L. (2007) *Nat. Rev. Mol. Cell Biol.* **8**, 284–295

5. Grant, P. A., Duggan, L., Côté, J., Roberts, J., Brownell, J. E., Candau, R., Ulba, R., Owen-Hughes, T., Allis, C. D., Winston, F., Berger, S. L., and Workman, J. L. (1997) *Genes Dev.* **11**, 1640–1650

6. Timmers, H. T., and Tora, L. (2005) *Trends Biochem. Sci.* **30**, 7–10

7. Wu, P. Y., Ruhlmann, C., Winston, F., and Schultz, P. (2004) *Mol. Cell* **15**, 199–208

8. Mohibullah, N., and Hahn, S. (2008) *Genes Dev.* **22**, 2994–3006

9. Brown, C. E., Howe, L., Sousa, K., Alley, S. C., Carrozza, M. J., Tan, S., and Workman, J. L. (2001) *Science* **292**, 2333–2337

10. Kuo, M. H., vom Baur, E., Struhl, K., and Allis, C. D. (2000) *Mol. Cell* **6**, 1309–1320

11. Henry, K. W., Wyce, A., Lo, W. S., Duggan, L. J., Emre, N. C., Kao, C. F., Pillus, L., Shilatifard, A., Osley, M. A., and Berger, S. L. (2003) *Genes Dev.* **17**, 2648–2663

12. Daniel, J. A., Torok, M. S., Sun, Z. W., Schiltz, D., Allis, C. D., Yates, J. R., 3rd, and Grant, P. A. (2004) *J. Biol. Chem.* **279**, 1867–1871
13. Ingvarsdottir, K., Krokan, N. J., Emre, N. C., Wyce, A., Thompson, N. I., Emili, A., Hughes, T. R., Greenblatt, J. F., and Berger, S. L. (2005) *Mol. Cell Biol.* **25**, 1162–1172
14. Bhaumik, S. R., and Green, M. R. (2002) *Mol. Cell Biol.* **22**, 7365–7371
15. Zhang, H., Kruk, J. A., and Reese, J. C. (2008) *J. Biol. Chem.* **283**, 27360–27368
16. Pascual-García, P., and Rodríguez-Navarro, S. (2009) *RNA Biol.* **6**, 141–144
17. Zhang, H., Kruk, J. A., and Reese, J. C. (2008) *J. Biol. Chem.* **283**, 27360–27368
18. Pascual-García, P., Govind, C. K., Queralt, E., Cuenca-Bono, B., Llopis, A., Chavez, S., Hinnebusch, A. G., and Rodríguez-Navarro, S. (2008) *Genes Dev.* **22**, 2811–2822
19. Kohler, A., Pascual-García, P., Llopis, A., Zapater, M., Posas, F., Hurt, E., and Rodríguez-Navarro, S. (2008) *Genes Dev.* **22**, 2811–2822
20. Koehler, A., Pascual-García, P., Llopis, A., Zapater, M., Posas, F., Hurt, E., and Rodriguez-Navarro, S. (2006) *Mol. Biol. Cell* **17**, 4228–4236
21. Biddick, R. K., Law, G. L., and Young, E. T. (2008) *PLoS One* **3**, e1436
22. Van Oevelen, C. J., van Teeffelen, H. A., van Werven, F. J., and Timmers, H. T. (2006) *J. Biol. Chem.* **281**, 4523–4531
23. Atanassov, B. S., Evrard, Y. A., Multani, A. S., Zhang, Z., Tora, L., Devys, D., Chang, S., and Dent, S. Y. (2009) *Mol. Cell* **35**, 352–364
24. Weake, V. M., Lee, K. K., Guelman, S., Lin, C. H., Seidel, C., Abmayr, S. M., and Workman, J. L. (2008) *EMBO J.* **27**, 394–405
25. Weake, V. M., Lee, K. K., Guelman, S., Lin, C. H., Seidel, C., Abmayr, S. M., and Workman, J. L. (2008) *EMBO J.* **27**, 394–405
26. Sterner, D. E., Belotserkovskaya, R., Deng, M., Sayre, M. H., Lieberman, P. M., and Berger, S. L. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11622–11627
27. Belotserkovskaya, R., Sterner, D. E., Deng, M., Sayre, M. H., Lieberman, P. M., and Berger, S. L. (2000) *Mol. Cell Biol.* **20**, 634–647
28. Wu, P. Y., and Winston, F. (2002) *Mol. Cell Biol.* **22**, 5367–5379
29. Lu, K. K., Swanson, S. K., Florens, L., Washburn, M. P., and Workman, J. L. (2009) *Epigenetics Chromatin* **2**, 2
30. Mischerikow, N., Spedale, G., Altelaar, A. F., Timmers, H. T., Pijnappel, W. W., and Knick, A. J. (2009) *J. Proteome Res.* **8**, 5020–5030
31. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) *Cell* **100**, 391–398
32. Wilson, A. C., Peterson, M. G., and Herr, W. (1995) *Genes Dev.* **9**, 2445–2458
33. Zhou, H., Spicuglia, S., Hsieh, J. J., Mitsu, D. I., Hsieh, T., Veenstra, G. J., Korsmeyer, S. J., and Stunnenberg, H. G. (2006) *Mol. Cell. Biol.* **26**, 2728–2735
34. Santos-Rosa, H., Kirmizis, A., Nelson, C., Bartke, T., Saksouk, N., Cote, J., and Kouzarides, T. (2009) *Nat. Struct. Mol. Biol.* **16**, 17–22
35. Duncan, E. M., Muratore-Schröder, T. L., Cook, R. G., Garcia, B. A., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2008) *Cell* **135**, 284–294
36. Parr, C. L., Keates, R. A., Brykza, B. C., Ogawa, M., and Yada, R. Y. (2007) *Yeast* **24**, 467–480
37. Zaman, S., Lippman, S. I., Zhao, X., and Broach, J. R. (2008) *Annu. Rev. Genet.* **42**, 27–81
38. Fournier, M. L., Paulson, A., Pavelka, N., Mosley, A. L., Gaudenz, K., Bradford, W. D., Glynn, E., Li, H., Sardiu, M. E., Fleharty, B., Seidel, C., Florens, L., and Washburn, M. P. (2010) *Mol. Cell. Proteomics* **9**, 271–284
39. Mason, D. A., Shulga, N., Undavali, S., Ferrando-May, E., Rexach, M. F., and Goldfarb, D. S. (2005) *FEMS Yeast Res.* **5**, 1237–1251
40. Deiss, L. P., Galkina, H., Berissi, H., Cohen, O., and Kimchi, A. (1996) *EMBO J.* **15**, 3861–3870
41. Röper, K. (2001) *Lab. Invest.* **81**, 149–158
42. Goulet, B., Baruch, A., Moon, N. S., Poitier, M., Sansregret, L. L., Erickson, A., Bogyo, M., and Nepveu, A. (2004) *Mol. Cell* **14**, 207–219
43. Hoke, S. M., Liang, G., Mutiu, A. I., Genereux, J., and Brandl, C. J. (2007) *BMC Biochem.* **8**, 16
44. Eisenmann, D. M., Chapron, C., Roberts, S. M., Doillard, C., and Winston, F. (1994) *Genetics* **137**, 647–657
45. Marques, M., Moro, D., Amorim, M. A., Almeida, T., Hohmann, S., Moradas-Ferreira, P., and Costa, V. (2006) *Microbiology* **152**, 3595–3605
46. Coffman, J. A., and Cooper, T. G. (1997) *J. Bacteriol.* **179**, 5609–5613
47. Hoke, S. M., Guzzo, J., Andrews, B., and Brandl, C. J. (2008) *BMC Genet.* **9**, 46