Attachment of the Ubiquitin-Related Protein Urm1p to the Antioxidant Protein Ahp1p

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Urm1p is a ubiquitin-related protein that serves as a posttranslational modification of other proteins. Urm1p conjugation has been implicated in the budding process and in nutrient sensing. Here, we have identified the first in vivo target for the urmylation pathway as the antioxidant protein Ahp1p. The attachment of Urm1p to Ahp1p requires the E1 for the urmylation pathway, Uba4p. Loss of the urmylation pathway components results in sensitivity to a thiol-specific oxidant, as does loss of Ahp1p, implying that urmylation has a role in an oxidative-stress response. Moreover, treatment of cells with thiol-specific oxidants affects the abundance of Ahp1p-Urm1p conjugates. These results suggest that the conjugation of Urm1p to Ahp1p could regulate the function of Ahp1p in antioxidant stress response in Saccharomyces cerevisiae.

An emerging theme in the regulation of protein function is the covalent attachment of one protein to another. An example of this type of modification is provided by ubiquitin, a 76-residue protein that can be attached to other proteins. In many cases, ubiquitination targets the modified proteins for degradation, but other regulatory effects of ubiquitination are also known. Ubiquitin is the founding member of a family of small modifier proteins present in most eukaryotes and known as ubiquitin-like proteins (Ubls) (20). As in the case of the ubiquitin pathway, the attachment of Ubls to targets requires a series of enzymatic steps that include an E1 (an activating enzyme), an E2 (a conjugating enzyme), and sometimes an E3 (protein ligase). Each Ubl is thought to be attached to other proteins via an isopeptide bond between the C terminus of the modifier and a lysine residue of a target protein (18). In contrast to ubiquitin, some of these Ubls do not appear to promote proteolysis but instead appear to alter the function or localization of the target (26, 31, 33, 36).

Most of the Ubls are attached to many targets and consequently are involved in the regulation of many processes. Probably the best-characterized ubiquitin-like pathway involves the attachment of SUMO to a rapidly growing set of targets. These substrates include RanGAP1, a Ran GDP-activating protein involved in nucleocytoplasmic transport (29, 36); IκBα, an inflammatory-response regulatory protein (10); PML (promyelocytic leukemia protein), whose fusion to the retinoic acid inflammatory-response regulatory protein (10); PML (promyelocytic leukemia protein), whose fusion to the retinoic acid receptor causes acute promyelocytic leukemia (29); and Cdc3p, Cdc11p, and Shs1p, proteins in the yeast Saccharomyces cerevisiae that are components of the septin ring involved in cytokinesis (25).

In addition to sumoylation, four other ubiquitin-like pathways have been identified in yeast (11, 20). The modifier of one of these protein conjugation systems is Urm1p (12); thus, the pathway is termed urmylation. The function of Urm1p conjugation is only beginning to be understood. The E1 for this pathway is Uba4p (12), but other components of the conjugation pathway, such as an E2 or an E3, have yet to be identified. Although urmylation has been linked to budding and invasion (17), no substrates are known for the urmylation pathway.

As a first step toward understanding the physiological role of the Urm1p conjugation pathway in S. cerevisiae, we were interested in identifying and characterizing substrate proteins that are modified by Urm1p. Here, we show that Urm1p is attached to Ahp1p (alkyl hydroperoxide reductase), a protein implicated in oxidative-stress protection in yeast (41). Furthermore, we identified a potential role for urmylation in oxidative-stress response.

MATERIALS AND METHODS

Strains, growth conditions, and plasmids. The yeast strains used in this study are listed in Table 1. Yeast transformations were performed as described previously (15). Haploid MATa nonessential yeast deletion strains were purchased from Research Genetics (Huntsville, Ala). Other gene deletions were constructed by PCR (4) using either the pRS (49) or pFA6a (34) plasmid series as templates. In all cases, the entire coding region was replaced with the indicated marker, and successful replacement was confirmed by PCR and phenotype when applicable.

Yeast strains were propagated using standard methods (47). Rich yeast medium containing 2% glucose (YPD) and synthetic yeast medium containing either 2% glucose (SD) or 2% galactose (SG) were prepared as described previously (47). Geneticin (Biovectors, Charlotte, N.C.) was added to the medium from a concentrated stock solution in 90% ethanol-10% Tween 20. To test for tert-butyl hydroperoxide (t-BOOH) and diamide sensitivities, cells were grown overnight in YPD medium, spotted on yeast extract-peptone-dextrose (YPED) plates containing either 0.4 mM t-BOOH (Sigma), 1 mM t-BOOH, 1 mM diamide (Sigma), or 1.3 mM diamide. The plates were monitored after 3 days of incubation at 30°C.

The PGal1::URA3-containing plasmids were constructed using Sfluoroacetic acid (Biovectors). Rapamycin (Sigma, St. Louis, Mo.) was added to the medium from a concentrated stock solution in 90% ethanol-10% Tween 20. To test for tert-butyl hydroperoxide (t-BOOH) and diamide sensitivities, cells were grown overnight in YPD medium, spotted on yeast extract-peptone-dextrose (YPED) plates containing either 0.4 mM t-BOOH (Sigma), 1 mM t-BOOH, 1 mM diamide (Sigma), or 1.3 mM diamide. The plates were monitored after 3 days of incubation at 30°C.

The PGal1::URM1 construct that bears the N-terminal extension MTS HHHHHHMHDDYKDIDDKMG5 contains His9 and FLAG tags (PGal1::URM1) has been described (17). The other plasmids used in this study, YCplac33clav4-75, pML40, pML41, pRS316::ADE8::CL44, pEGH PGal1::GFP-STD-His6X, and pEGH PGal1::GFP-STD-His6X-AHP1, were described previously (9, 35, 39, 55).

Immunoblot analysis of whole-yeast lysates and antibodies. Immunoblot analysis of yeast lysates was performed as previously described (17). When indicated, strains were treated with 1.5 mM diamide or 1.3 mM t-BOOH for 4 h prior to

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were searched using SEQUEST software against the translated open reading frame of Urm1p containing at least two peptides with Xcorr scores above 2.0. The resulting peptide matches were scored by SEQUEST, and protein identifications were considered valid if the identified protein contained at least two peptides with Xcorr scores above 2.0. A number of peptides were confirmed by tandem mass spectrometry (MS/MS).

**RESULTS**

In an attempt to identify substrates of the urmylation pathway, we constructed a strain that expressed His
tagged URM1 as its only copy of URM1. Extracts from cells expressing HURM1 were incubated with Talon resin. Bound proteins were eluted with imidazole and bound to anti-FLAG agarose. When proteins bearing both tags were eluted with loading buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by Western blotting, the banding pattern was similar to the pattern of Urm1p conjugates recognized by the Urm1p antibody.
Peroxiredoxins are a class of enzymes, present in most organisms, that promote the elimination of H$_2$O$_2$ and alkyl hydrogen peroxides as a cellular defense against oxidative stress (8, 37, 40). In vitro assays show that Ahp1p preferentially reduces alkyl hydrogen peroxides, such as $t$-BOOH, while other peroxiredoxins preferentially reduce H$_2$O$_2$ (24, 42). Furthermore, loss of Ahp1p results in sensitivity of cells to the oxidant (32).

Recently, Ahp1p was identified as a peroxiredoxin (24, 32, 52). Peroxiredoxins are a class of enzymes, present in most organisms, that promote the elimination of H$_2$O$_2$ and alkyl hydrogen peroxides as a cellular defense against oxidative stress (8, 37, 40). In vitro assays show that Ahp1p preferentially reduces alkyl hydrogen peroxides, such as $t$-BOOH, while other peroxiredoxins preferentially reduce H$_2$O$_2$ (24, 42). Furthermore, loss of Ahp1p results in sensitivity of cells to the oxidants $t$-BOOH, an organic peroxide, and diamide, a thiol oxidant (32).

**Urmylation Pathway Components.** To further support the idea that Urm1p was conjugated to Ahp1p, we performed Western analysis using the anti-Urm1p antibody on strains lacking URM1, UBA4, and AHP1. Loss of AHP1 resulted in the disappearance of the ~32-kDa protein, but did not affect the abundance of the ~75-, ~80-, ~89-, and 125-kDa polypeptides (Fig. 3, lane ahp1Δ). However, loss of URM1 and UBA4 eliminated the ~32-kDa protein, as well as many other polypeptides of higher molecular mass (Fig. 3, lanes urm1Δ and ube4Δ). Collectively, these results support the conclusion that Ahp1p is one of the several targets of the urmylation pathway.

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**Urm1p is conjugated to Ahp1p.** In order to confirm that Urm1p was interacting with Ahp1p, we used a GST-tagged version of Ahp1p. Purification of Ahp1p from wild-type extracts and immunoblotting with Urm1p-specific antibodies indicated that a 32-kDa species of Ahp1p contained Urm1p (Fig. 2, right lane). These bands were not detected in extracts from cells expressing GST alone (Fig. 2, left lane). Intriguingly, either Urm1p itself or Ahp1p may undergo posttranslational modification, since the conjugated product appears as a doublet.

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of Ahp1p causes a defect in invasion. While deletion of UBA4 and URM1 rendered cells unable to invade agar under starvation conditions (data not shown), deletion of AHP1 did not affect invasive growth. Therefore, Ahp1p does not appear to play a role in invasion.

**Potential role for Ahp1p in TOR signaling.** Loss of the urmylation pathway also resulted in hypersensitivity to rapamycin (a macrocyclic antibiotic) (17). Rapamycin inhibits Tor1p and Tor2p, ultimately resulting in cellular responses characteristic of nutrient deprivation through a mechanism involving translational and transcriptional arrest (3, 19, 27, 30). Since Ahp1p appears to be a target of urmylation, we tested whether ahp1 mutants are hypersensitive to rapamycin and if the mutations exhibited genetic interaction with TOR pathway mutations. A gln3Δ mutant, which is defective for a GATA-type transcription factor regulated by the TOR kinases and by the Ure2p repressor (5, 7, 44), served as a rapamycin-resistant control strain. A ure2Δ mutant served as a control for rapamycin sensitivity. We found that ahp1 null mutants were unable to grow after 5 days on medium containing rapamycin (data not shown). Moreover, even if the ahp1 null mutants were carrying a wild-type copy of TOR2 on a plasmid, the cells were still unable to grow after 5 days on medium containing rapamycin (Fig. 4A). The sensitivity to rapamycin was not due to a reduction in protein synthesis or a general drug sensitivity, because the ahp1 null mutants were not sensitive to cycloheximide (data not shown). To verify that the sensitivity of these mutants to rapamycin reflected inactivity of the TOR pathway, we measured the rapamycin sensitivities of ahp1Δ mutants carrying a plasmid-borne TOR2S1972I (TOR2-1) allele (35). This allele of TOR2 has previously been shown to confer rapamycin resistance on many sensitive mutants. We found that all the double mutants were no longer sensitive to rapamycin (Fig. 4B). In contrast, the ahp1Δ mutants carrying a wild-type version of TOR2 on a plasmid were still sensitive to rapamycin (data not shown). These results suggest that, like the urmylation pathway mutants, ahp1 mutants are hypersensitive to rapamycin because of inhibition of TOR pathway signaling.

**Urmylation is involved in oxidative stress.** Ahp1p is believed to function as an antioxidant specific for t-BOOH and the thiol oxidant diamide (24, 32, 42). We therefore tested whether the loss of the urmylation pathway results in sensitivity to either t-BOOH or diamide. The urm1 and the uba4 mutant strains were sensitive to diamide (Fig. 5B). In fact, the loss of URM1 or UBA4 caused cells to be more sensitive to diamide than did loss of AHP1. In contrast, although the ahp1 null mutant strain was hypersensitive to t-BOOH compared to the wild-type control, the urm1 and the uba4 mutant strains were not sensitive to t-BOOH (Fig. 5A). Taken together, these results suggest that the urmylation pathway has an antioxidant role specifically toward diamide in vivo. Furthermore, these results also suggest that the conjugation of Urm1p to Ahp1p affects the antioxidant role of Ahp1p toward diamide but not toward t-BOOH.

Given that Ahp1p is urmylated and controls sensitivity to t-BOOH and to diamide, we asked whether the state of Ahp1p urmylation was modulated by treatment with either of these oxidants. Urmylation was modulated by treatment with either of these oxidants. Furthermore, these results also suggest that the conjugation of Urm1p to Ahp1p affects the antioxidant role of Ahp1p toward diamide but not toward t-BOOH.
agents. We observed a modest increase in the Ahp1p-Urm1p conjugate when cells were treated with diamide (Fig. 6). In contrast, the Ahp1p-Urm1p conjugate was absent from cells treated with t-BOOIH. Whether urmylation of Ahp1p is blocked by t-BOOIH or Ahp1p is degraded under these conditions cannot be determined from these data, though the former possibility seems more likely. Nonetheless, the salient point is that urmylation of Ahp1p is differentially affected by diamide and t-BOOIH, concordant with the observation that the loss of urmylation causes a differential effect on sensitivity to these agents.

**DISCUSSION**

In order to investigate the role of the Urm1p conjugation pathway, we sought to identify proteins modified by Urm1p using MS. This effort identified the first target for the urmylation pathway, the antioxidant protein Ahp1p. We showed that the conjugation of Urm1p to Ahp1p requires the E1 of the urmylation pathway, Uba4p. Moreover, we found that the loss of the urmylation pathway components causes cells to be sensitive to diamide. These results suggest a role for the urmylation pathway in antioxidant activity specific for diamide.

**Conjugation of Urm1p to Ahp1p.** Only a single copy of Urm1p appears to be attached to each Ahp1p polypeptide. Two species of urmylated GST-HisX6-Ahp1p were detected by Western blotting. One is the expected size for urmylated GST-HisX6-Ahp1p. The second band is not sufficiently larger to contain a second Urm1p moiety. This result suggests that either Ahp1p or Urm1p is posttranslationally modified (Fig. 2). Since our previous results suggest that there is a higher-molecular-weight Urm1p species, it is possible that Urm1p is the protein that is modified (17).

Although we have not identified which of the 14 lysines on Ahp1p is modified by Urm1p, two results support the idea that Ahp1p is a target and not an enzyme of the urmylation pathway. First, although the Ahp1p-Urm1p conjugate is detected only when 20 mM N-ethylmaleimide is present during cell lysis, these samples were resuspended in loading buffer that contained 2-mercaptoethanol (βME) (17)(Fig. 3). Sample buffer containing this reducing agent should disrupt the Urm1 thiols esters of E1 or E2, whereas Urm1-protein conjugates would be resistant to βME (21). Because the Urm1p-Ahp1p product is present when treated with βME, it seems likely that Ahp1p is a target of the urmylation pathway. Second, loss of AHP1 affects the presence of only the 32-kDa Urm1p-Ahp1p conjugate, not other urmylated targets (Fig. 3). If Ahp1p functioned as an E2 or an E3 for the urmylation pathway, then the loss of Ahp1p would be expected to affect at least some of these other bands. Together, our results suggest that Ahp1p is a target of the urmylation pathway.

**Possible functions for Urm1p attachment to Ahp1p.** When cells are exposed to elevated levels of reactive oxygen species, they suffer oxidative stress. Oxidative stress can lead to DNA damage, lipid peroxidation, and protein oxidation (22, 50). The altered diamide tolerance phenotype seen in the ahp1 null mutant is believed to be related to the generation of lipid hydroperoxides upon exposure to the oxidant diamide (32). Similarly, mutations in either URM1 or UBA4 cause cells to become hypersensitive to diamide. Our results therefore suggest a potential role for the urmylation pathway in an antioxidant activity specific for diamide. In keeping with this possi-

![FIG. 5. Sensitivities of the urmylation pathway mutants to oxidative stress. (A) Loss of the urmylation pathway does not result in sensitivity to t-BOOIH. Strains BY4741 (wild type [WT]), SY4119 (ahp1Δ), SY3839 (urm1Δ), SY3840 (uba4Δ), SY4122 (ahp1Δ urm1Δ), SY4123 (ahp1Δ uba4Δ), and SY4124 (urm1Δ uba4Δ) were grown to mid-log phase in YEPD at 30°C. A serial dilution (1/5) was performed starting with 10,000 cells. Cells were spotted onto either YEPD plus 0.4 mM t-BOOIH (left), YEPD plus 1 mM t-BOOIH (middle), or 1.5 mM t-BOOIH (right) and grown for 3 days at 30°C. (B) Loss of Ahp1p and the urmylation pathway results in diamide sensitivity. Strains BY4741 (wild type), SY4119 (ahp1Δ), SY3839 (urm1Δ), SY3840 (uba4Δ), SY4122 (ahp1Δ urm1Δ), SY4123 (ahp1Δ uba4Δ), and SY4124 (urm1Δ uba4Δ) were grown to mid-log phase in YEPD at 30°C. A serial dilution (1/5) was performed starting with 10,000 cells. Cells were spotted onto either YEPD (left), YEPD plus 1 mM diamide (middle), or 1.3 mM diamide (right) and grown for 3 days at 30°C.

![FIG. 6. The Ahp1p-Urm1p conjugate is affected by diamide and t-BOOIH treatment. Strain SY3357 (wild type) was grown to mid-log phase in YEPD at 30°C. Cells were then grown in YEPD at 30°C in the presence of 1.5 mM diamide or 1.3 mM t-BOOIH or without drug treatment for 4 h. The lysates were analyzed by SDS-PAGE and immunoblot analysis using affinity-purified polyclonal anti-Urm1p antibodies and monoclonal antibodies to Dpm1p.](http://ec.asm.org/).
bility, treatment of cells with diamide causes an increase, albeit a modest one, in the amount of urmylated Ahp1p. Interestingly, the loss of the urmylation pathway components causes cells to be more sensitive to diamide than does the loss of Ahp1p. One possible explanation for the difference in diamide sensitivity may be that Urm1p interacts with another antioxidant enzyme(s). In support of this possibility, previous results indicated that different peroxiredoxins in yeast might have redundant and nonredundant functions (42, 54).

We also identified a potential interaction between Ahp1p and the TOR pathway. As seen for loss of URM1 or UBA4, the loss of AHP1 confers sensitivity to rapamycin. Since loss of AHP1 confers rapamycin sensitivity, it might be an enhancer of TOR signaling or of a cellular process regulated by TOR. Recent results suggest that the TOR pathway may play a role in oxidative-stress response. In Schizosaccharomyces pombe, tor1+ is required for response to oxidative stress, as well as other stresses (53). Therefore, Urm1p targeting of Ahp1p may provide a link between the TOR pathway and oxidative-stress response in S. cerevisiae.

Implications of urmylation and oxidative-stress response in mammals. Damage to cells caused by oxidative stress has been implicated in aging (51), neurodegenerative diseases (6), diabetes (28), and cancer (2). In mammals, reactive oxygen species are a well-established signal for regulation of transcription factors, such as NF-kB (nuclear factor kB), Ap1 (activator protein 1), and p53 (tumor suppressor) (1, 13, 23, 38). In higher plants, as well as animals, reactive oxygen species are also established signals in response to wound healing (43, 46). Thus, a growing area of research is focused on trying to understand the mechanisms that protect cells from oxidative stress or promote cellular recovery. Potential homologues for Ahp1p have been identified in plants, animals, fungi, and prokaryotes (52). Sequence similarity with these other potential antioxidant enzymes is highest around a putative catalytic active site that may be important for the peroxidase function (cysteine 62 in Ahp1p) (52). Since there also appears to be a homologue to Urm1p in mammals, perhaps urmylation of Ahp1p is important in oxidative-stress response in mammals. Further characterization of Ahp1p and the attachment of Urm1p is necessary to determine if this conjugate is involved in an antioxidant mechanism and/or other functions in S. cerevisiae.

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