Determinants of the Ubiquitin-mediated Degradation of the Met4 Transcription Factor*

Received for publication, January 3, 2006, and in revised form, February 23, 2006 Published, JBC Papers in Press, February 23, 2006 DOI 10.1074/jbc.M600037200

Alexandra Menant1, Peggy Baudouin-Cornu1,2, Caroline Peyraud3, Mike Tyers4, and Dominique Thomas1,5

From the 1Centre de Généétique Moléculaire, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, France, 2Cytomics Systems SA, Bâtiment 5, 1 Avenue de la Terrasse, 91190 Gif-sur-Yvette, France, and 3Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada MSG 1X5

In yeast, the Met4 transcription factor and its cofactors Cbf1, Met28, Met31, and Met32 control the expression of sulfur metabolism and oxidative stress response genes. Met4 activity is tuned to nutrient and oxidative stress conditions by the SCF\textsuperscript{Met30} ubiquitin ligase. The mechanism whereby SCF\textsuperscript{Met30}-dependent ubiquitylation of Met4 controls Met4 activity remains contentious. Here, we have demonstrated that intracellular cysteine levels dictate the degradation of Met4 \emph{in vivo}, as shown by the ability of cysteine, but not methionine or \(S\)-adenosylmethionine (AdoMet), to trigger Met4 degradation in an \textit{str4A} strain, which lacks the ability to produce cysteine from methionine or AdoMet. Met4 degradation requires its nuclear localization and activity of the 26 S proteasome. Analysis of the regulated degradation of a fully functional Met4-Cbf1 chimera, in which Met4 is fused to the DNA binding domain of Cbf1, demonstrates that elimination of Met4 \emph{in vivo} can be triggered independently of both its normal protein interactions. Strains that harbor the Met4-Cbf1 fusion as the only source of Cbf1 activity needed for proper kinetochore function exhibit high rates of methionine-dependent chromosomal instability. We suggest that SCF\textsuperscript{Met30} activity or Met4 utilization as a substrate may be directly regulated by intracellular cysteine concentrations.

The temporal and spatial control of transcription requires a battery of different factors, including transcription activators/repressors, co-activators/repressors, general transcription factors, chromatin-modifying and -remodeling enzymes, and the multisubunit enzyme RNA polymerase II. Diverse biochemical events dictate interaction of RNA polymerase II with gene regulatory sequences. Protein phosphorylation controls interactions between many factors, while a complex code of histone acetylation, methylation, and ubiquitylation modifies chromatin accessibility (2). Finally, the abundance and/or activity of many transcription-associated factors is dynamically controlled by the ubiquitin-proteasome system (3, 4).

Conjugation of ubiquitin to substrate proteins is achieved through a cascade of E1, E2, and E3 enzymes, which activate and then serially transfer ubiquitin onto substrate lysine residues (5). The best understood function of ubiquitylation is to target proteins for destruction by the 26 S proteasome, a large compartmentalized protease particle that recognizes the polyubiquitin tag (6). More recently, non-proteolytic functions have been ascribed to ubiquitin conjugation, including targeting to different subcellular compartments and allosteric control of enzymatic events (7). How ubiquitylation leads to different outcomes in different contexts is not fully understood.

The pervasive role of the ubiquitin proteasome pathway in the regulation of transcription has gradually emerged from numerous studies. RNA polymerase II is polyubiquitylated in response to DNA damage, whereas histone monoubiquitylation is associated with activation of transcription (8, 9). Surprisingly, the proteasome itself regulates different steps in transcription and is recruited to active gene regions (10). Finally, the regulated ubiquitin-dependent degradation of transcriptional activators allows the cells to dynamically control the expression of target genes (3). Moreover, an intriguing correlation between activator potency and instability suggests that ubiquitylation of transcription factors may be directly linked to transcriptional activation (4, 11). The most potent class of transcriptional activators, those containing transcription activation domains rich in acidic residues, are by far the most unstable; strikingly, such transcription activation domains can function as degrons that target heterologous proteins for degradation (12, 13). Degradation of transcription factors may thus be coupled to assembly of active transcriptional complexes on promoter DNA (14, 15).

In addition to proteasomal-dependent degradation, non-proteolytic regulation of transcription factor activity by ubiquitylation has recently been described. The first and best documented example of this mechanism is the yeast transcriptional activator Met4, which governs the MET gene network responsible for the biosynthesis of the sulfur-containing amino acids, methionine and cysteine (16). Met4 is the essential substracte of the SCF\textsuperscript{Met30} ubiquitin ligase, a multiprotein E3 complex that uses the F-box protein subunit Met30 to recruit Met4 for ubiquitylation by the core SCF complex, in conjunction with the E2 enzyme Cdc34 (17, 18). Met4 is recruited to promoter DNA by one of two distinct sets of cofactors that bind different elements in MET gene regulatory regions, either the Met28-Cbf1 complex or the complex Met31-Met32 (19, 20). In addition to its role in facilitating activation of some MET genes, Cbf1 is a component of the core kinetochore complex that binds the CDEI of the yeast centromere (21, 22).

Upon environmental changes, Met4 can be either rapidly ubiquitylated and degraded (23) or ubiquitylated and inactivated in the absence of degradation (24). These alternative fates of ubiquitylated Met4 forms

---

* This work was supported in part by funds from the Centre National de la Recherche Scientifique and the Association de la Recherche sur le Cancer (to D. T.) and by grants from the National Cancer Institute of Canada and the Canadian Institutes of Health Research (CIHR) (to M. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 Supported by a thesis fellowship from the Ministère de la Recherche et de l’Enseignement Supérieur and by the Fondation pour la Recherche Médicale.
2 Supported by the Leukemia and Lymphoma Society of America. Present address: Service de Biochimie et Génétique Moléculaire, CEA/SAclay, 91191 Gif-sur-Yvette, France.
3 Present address: Institut Pasteur, 25 Rue du Docteur Roux, 75015 Paris, France.
4 Supported by a Canada Research Chair in Functional Genomics and Bioinformatics.
5 To whom correspondence should be addressed. Tel.: 33-1-69-82-42-66; Fax: 33-1-69-82-42-38; E-mail: dthomas@cytomics.fr.

---

6 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GFP, green fluorescence protein; HA, hemagglutinin; AdoMet, adenosylmethionine.
depend upon the growth conditions: in minimal media Met4 is ubiquitylated and degraded in response to methionine excess, whereas in rich media Met4 is oligo-ubiquitylated but remains stable (25). In the latter growth condition, oligo-ubiquitylated Met4 is not recruited to the SAM genes, which are required for production of S-adenosylmethionine, an unstable metabolite that is not present in rich medium. Thus, ubiquitylation appears to not only regulate Met4 by distinct mechanisms but also to control the differential recruitment of Met4 to distinct target promoters. Despite multiple lines of evidence that SCF<sup>Met30</sup>-dependent ubiquitylation of Met4 leads to Met4 degradation specifically in minimal media (23, 25), the degradation of Met4 under minimal conditions has recently been disputed (26). To settle this discrepancy, we have investigated the determinants of Met4 degradation specifically in minimal media (23, 25), the degradation-dependent and -independent mechanisms.

**EXPERIMENTAL PROCEDURES**

**Yeast Culture**—Saccharomyces cerevisiae strains used in this study are listed in Table 1. All strains are isogenic with the wild-type strain W303 (provided by R. Rothstein). Standard yeast media and minimal B medium were prepared as described by Chérest and Surdin-Kerjan (27). Minimal B medium is a synthetic medium without sulfur sources (27).

For methionine repression, cells were grown in B medium supplemented with 0.05 mM L-methionine to early log phase and transferred to a B medium without sulfur source for 1 h before a high concentration of L-methionine (1 mM) was added. For derepression, cells were first grown in B medium containing a repressive amount of L-methionine to early log phase and then transferred to B medium without sulfur amino acids. Transformation was by the lithium acetate method (28).

**Plasmid Construction**—The plasmid pGAL1-GFP-MET4<sub>Delt</sub> that encodes different GFP-Met4 derivatives expressed from the GAL1 promoter was created by cloning the fragments EcoRI-BamHI of plasmid pLexMet4<sub>Delt</sub> (29) into the plasmid pGAL306GFP (23) that had been digested by EcoRI and BamHI. The plasmid pGAL1-GFP-MET4<sub>Delt</sub> was constructed by amplifying MET4 nucleotides 548–1200 from plasmid pMET4<sub>Delt</sub> –1 (29) with oligonucleotide primers containing XhoI restriction sites and cloning into the Xhol site of pLexMET4<sub>Delt</sub>. The EcoRI-BamHI fragment of the resulting plasmid was then cloned into the corresponding sites of pGAL306GFP. The above plasmids were linearized by SstI to direct integration into the chromosomal URA3 locus in a met4<sup>Delt</sup>-TRPI strain (CC849 –1A).

The Met4-Cbf1 chimera comprises the entire Met4 protein with the exception of the basic leucine zipper domain fused to the basic helix-loop-helix DNA binding domain of Cbf1. The plasmids were constructed as follows: pGAL136MET4-CBF1, which allows the expression of the Met4-Cbf1 chimera from the GAL1 promoter, was created by inserting the Ccl-ClaI fragment of pMET4 –4 (30) treated with Klenow enzyme into pGAL316 (23) that had been digested by BamHI and EcoRI, treated with Klenow, and dephosphorylated. The resulting plasmid was digested with Spel and NotI and in-frame ligated with a Cbf1-encoding EcoRI-NotI fragment from plasmid Ref. 23.
Ubiquitin-mediated Degradation of Met4

pET28Chf1Δ1-209 (19) via a 16-base pair linker with SpeI and EcoRI compatible ends. pProMET4-CBF1, which allows the expression of the Met4-Cbf1 chimera from the endogenous MET4 promoter, was created by inserting the EcoRI-NotI fragment from pGAL316MET4-CBF1 into the pProMET4 plasmid (containing the MET4 promoter region, (29)) that had been digested by EcoRI/NotI and dephosphorylated. To direct the homologous replacement of the MET4 chromosomal locus, we cloned a HindIII-HindIII fragment that contains the MET4 terminator region of pMET4 – 4 into pProMET4-CBF1 that had been digested by HindIII and dephosphorylated. The CiaI-NotI fragment of the resulting plasmid pProMet4-Cbf1 was isolated, non-yeast nucleotides were removed by brief Bal31 digestion, and the resulting purified fragment was used to transform the CC849 – 8A strain.

Chromatin Immunoprecipitation—Cross-linked chromatin preparation and immunoprecipitation were performed as described previously (25), except that cells were fixed with 1.4% formaldehyde and disrupted using the Fastprep apparatus (Qbiogene) instead of vortexing. Rabbit polyclonal Met4 antisera against full-length Met4 produced in insect cells was used for immunoprecipitation (25). Immunoprecipitated and total DNA samples were purified using the Quiapquick PCR purification kit (Qiagen) and analyzed by quantitative real-time PCR performed with the Light Cycler System (Roche Applied Science). Input DNA was diluted to 1/5 and 1/25. Immunoprecipitated and total DNAs were subjected to 40 cycles of PCR using the FastStart DNA Master SYBR Green I reaction mix (Roche Applied Science). The occupancy level at a given promoter was defined as the ratio of immunoprecipitated DNA over total DNA for each PCR product. The higher occupancy level measured was arbitrarily set to 100, and all other values were represented relative to this standard.

Protein and RNA Analysis—Total proteins were extracted by a 20% cold trichloroacetic acid procedure (23) using a Fastprep apparatus (Qbiogene). Samples were separated on a 7.5% acrylamide gel, transferred to a nitrocellulose membrane (Optitran; Schleicher & Schuell), and probed. Anti-HA antibody (12CA5; Roche Applied Science) and peroxidase-conjugated anti-mouse antibody (Sigma) were used at 1:1,000 dilution. Anti-Met4 polyclonal antibody was used at 1:2,000 and peroxidase-conjugated anti-mouse antibody (Sigma) were used at a 1:10,000 dilution. Detection was by Supersignal chemiluminescent substrate (Pierce). Equal loading was established by detection with a lysis/ RNA synthetase antibody. Total cellular RNA was extracted from yeast by the hot phenol method, separated, and probed as described (23).

Fluorescent Microscopy and Cytometry—GFP-Met4 fusion protein fluorescence was monitored in living cells on a Zeiss Axioplan 2 fluorescence microscope using a Zeiss GFP filter. All the images were collected with a Roper Photometrics CoolSNAP HQ CCD camera using identical settings and analyzed with the Metamoph software (Universal Imaging Corp., Downingtown, PA). Immediately before observation, cells were harvested by a brief centrifugation, washed and suspended in Dabcbo (Sigma) buffer. Nuclei were stained using the dye HOECHST number 33342 (Sigma). Levels of the GFP-Met4 fusion protein were assessed by quantitative fluorescence of live cells using a BD Biosciences FacsScalibur flow cytometer.

Metabolic Sulfur Analysis—Yeast cells were grown at a cell density of 10^6 cells/ml in B medium containing 0.2 mM homocysteine as sulfur source. After filtration, cells were transferred to B medium containing 0.05 mM sulfate and 4 μCi/ml of radioactive sulfate. At the indicated time, a 5-ml aliquot of cells was harvested by a brief centrifugation, washed two times with 1 mM cold sulfate, boiled, and analyzed by thin layer chromatography.

RESULTS

Met4 Elimination Depends on the Trans-sulfuration Pathway—To elucidate the mechanisms that underlie the proteasome-dependent degradation of Met4, we first investigated the cellular signal that might trigger the elimination of Met4. High concentrations of the three predominant sulfur-containing compounds (1 mM 1-methionine, 0.2 mM S-adenosylmethionine (AdoMet), and 1 mM L-cysteine; Fig. 1A) were equally capable of repressing MET gene expression in wild-type cells grown in minimal medium (Fig. 1B). In contrast, in str4Δ mutants that lack a functional cystathionine-B synthase and are thus unable to convert either methionine or AdoMet into cysteine, only high levels of cysteine were able to cause full MET gene repression (Fig. 1B). To determine whether cysteine caused Met4 elimination, we monitored the stability of a GFP-Met4 fusion protein expressed from the endogenous MET4 promoter in cells exposed to either methionine or cysteine. In wild-type cells, the GFP-Met4 fluorescence signal disappeared with similar kinetics in response to either amino acid, whereas in str4Δ cells GFP-Met4 disappeared only in response to cysteine (Fig. 1C). To corroborate these results, we determined the stability of an HA epitope-tagged version of Met4 expressed from the endogenous MET4 promoter. The changes in abundance of HA-Met4 in wild-type versus strΔ cells exposed to either methionine or cysteine paralleled effects on MET gene expression and GFP-Met4 (Fig. 1D). The SCFMet30-dependent elimination of Met4 in response to methionine thus requires an increase in intracellular cysteine, which arises from the conversion of methionine into AdoMet and subsequent metabolism by the methyl cycle and the trans-sulfuration pathway (Fig. 1A). Glutathione, the most abundant cysteine-containing peptide in the cell, was not required for Met4 regulation because cysteine was able to promote rapid GFP-Met4 elimination in a gsh1Δ mutant that lacks γ-glutamylcysteine synthase and is thus unable to convert cysteine into glutathione (Fig. 1E).

Met4 Degradation Requires Nuclear Localization—Met30 is localized within the nucleus, and its correct compartmentalization is required for its function (32). Nuclear localization of Met4 should thus be required for its degradation. To identify nuclear localization signals in Met4, we constructed several Met4 deletion derivatives and monitored the subcellular localization of each derivative fused to GFP. This analysis identified three sequences within Met4 that were important for its subcellular localization (Fig. 2, A and B, data not shown). Deletion of the Met4 carboxyl-terminal region (404–589; deletions Δ7 and Δ8) caused cytoplasmic localization, implying the existence of an NLS in this region. Unexpectedly, larger deletions that removed additional adjacent residues (189–589; deletions Δ9 and Δ10) caused relocalization to the nucleus, suggesting that the 309–404 region of Met4 contains residues that help restrict Met4 to the cytoplasm. The cytoplasmic localization of severely truncated derivative GFP-Met4Δ11 suggested that Met4 contains a second NLS in the 78–189 region, which is close to the Met4...
A final GFP-Met4 derivative that was devoid of both potential NLS sequences, Met4\textsubscript{2NLS}, displayed mainly cytoplasmic localization (Fig. 2C). Interestingly, the sequence that helps restrict Met4 to the nucleus perfectly overlaps with the Met4 auxiliary domain, a sequence previously identified as being required for the full transcription activation potency of Met4 (29).
FIGURE 2. Met4 degradation is dependent upon its nuclear localization. A, schematic representation of the internal in-frame deletions of Met4. Plasmids encoding the different GFP-Met4 derivatives were integrated at the URA3 locus in a met4::TRP1 strain. B, localization of the GFP-Met4-derivative proteins. GFP-Met4 derivatives were expressed from the GAL1 promoter, nuclei stained with the HOECHST 33342 dye (Hoe), and fluorescent images acquired. C, Met4 that lacks both NLS sequences is localized in the cytoplasm. The fusion protein GFP-Met4Δ2NLS was expressed from the GAL1 promoter and fluorescent images acquired. D, methionine triggers destabilization of a GFP-Met4 fusion protein localized within the nucleus. GFP-Met4 (strain C301; met4::TRP1, ura3::pGAL1-GFP-MET4::URA3) and GFP-Met4Δ7 (strain CD307; met4::TRP1, ura3::pGAL1-GFP-MET4Δ7::URA3) fusion proteins were expressed from the GAL1 promoter and then repressed by the addition of glucose, either in the absence (−Met) or presence of 1 mm -methionine (+Met). Fluorescence images were acquired at the indicated times.
Ubiquitin-mediated Degradation of Met4

To determine whether Met4 degradation was dependent on its nuclear localization, we investigated the stability of the GFP-Met4Δ7 derivative (deletion of residues 470–589), which leaves both the Met30-interacting region and the known ubiquitylation site (lysine 163) intact (26). The stability of GFP-Met4Δ7 and wild-type GFP-Met4 fusion proteins was analyzed in the presence and the absence of high methionine after repression of the GAL1 promoter from which each was expressed. Wild-type GFP-Met4 was rapidly depleted in the presence of methionine, whereas much of the GFP-Met4Δ7 derivative was still present 60 min after methionine addition (Fig. 2D). Close inspection revealed that GFP-Met4Δ7 fluorescence was restricted to the cytoplasm in cells exposed to high methionine while it was present in both the nucleus and the cytoplasm in the absence of methionine. This result suggested that only nuclear localized GFP-Met4Δ7 was actively degraded. Immunoblot analysis revealed that the Met4Δ7 protein was less destabilized upon methionine exposure than wild-type Met4 (data not shown).

Met4 Degradation Is Dependent on Proteasome Activity—We next demonstrated that the loss of GFP-Met4 fluorescence signal in response to methionine indeed resulted from the proteasome-mediated degradation of the fusion protein and not, for instance, nonspecific disruption of the GFP moiety. To this end, the stability of the wild-type GFP-Met4 expressed from the GAL1 promoter was analyzed in the presence of Mg132 (carbobenzoxyl-leucinyl-leucinyl-leucinal), a peptide aldehyde inhibitor of proteasome (33). The concurrent addition of MG132 and methionine to minimal medium prevented the disappearance of GFP-Met4 that was observed when promoter shutoff was performed in the presence of methionine alone (Fig. 3, A and B). Moreover, both fluorescence microscopy and quantitative flow cytometry assays showed that the inhibition of fluorescence depletion was proportional to the concentration of added MG132 (Fig. 3, A and B).

We further ascertained that the disappearance of GFP-Met4 fluorescence was a result of ubiquitin-mediated degradation by analysis of a GFP-Met4Δ12 fusion protein, which lacks residues 79–180, a region that spans the activation domain and includes lysine 163, the sole ubiquitylation site on Met4 (26). The fluorescent signal emanating from GFP-Met4Δ12 was stable in the presence of methionine (Fig. 3C). An analogous HA-Met4Δ12 derivative was also not significantly destabilized upon methionine exposure as compared with wild-type Met4 (Fig. 3D). The HA-Met4Δ12 protein was only faintly post-translationally modified, consistent with a primary defect in ubiquitylation. Based on these results, we conclude that the rapid depletion of GFP-Met4 in live cells exposed to high methionine indeed arises from its ubiquitin-dependent elimination by the proteasome.

A Met4-Cbf1 Chimera Protein Drives Methionine Synthesis—Met4 is recruited to DNA by two distinct sets of cofactors, Cbf1-Met28 and Met31-Met32 (19). In contrast to Met4, the Cbf1, Met28, Met31, and Met32 DNA-binding proteins were shown to be stable proteins (23). Rather than proteasome-mediated elimination of Met4, it has been postulated that Met4 ubiquitylation causes ejection of the Cbf1 DNA binding subunit from promoter DNA without apparently affecting Met4 occupancy (24). To test this model and assess the possible effects of local protein interactions on Met4 degradation, we constructed a Met4-Cbf1 chimera protein in which the basic leucine zipper domain of Met4 that tethers it to other factors was replaced by the basic helix-loop-helix DNA binding domain of Cbf1 (see “Experimental Procedures”). The functionality of this Met4-Cbf1 chimera was assayed by testing its ability to relieve the methionine auxotrophy of single met4Δ and cbf1Δ and double met4Δ,cbf1Δ mutant strains. The expression of the Met4-Cbf1 chimera from a GAL1 promoter restored the methionine prototrophy of both the single and the double met4Δ,cbf1Δ mutant cells (Fig. 4, A and B). We then constructed strains in which the MET4-CBF1 chimera was expressed from the endogenous MET4 promoter. Expression of chimera from the chromosomal MET4 locus rescued the methionine auxotrophy of the double met4Δ,cbf1Δ deletion (data not shown). The kinetics of sulfate reduction was then assessed in wild-type and met4Δ:MET4-CBF1 cells by measuring the incorporation of radioactive sulfate into methionine and glutathione. Both compounds accumulated at similar rates in both strains (Fig. 4C). The Met4-Cbf1 chimera thus recapitulates the collective activities of Met4 and Cbf1 in methionine synthesis.

Methionine-triggered Elimination of a Met4-Cbf1 Chimera—To address how the activity of the Met4-Cbf1 chimera is regulated in cells exposed to high methionine, we analyzed the kinetics of both MET gene derepression and repression in cells whose MET4 chromosomal gene was replaced by the MET4-CBF1 allele. Cells were grown in minimal B medium in the presence of high methionine, shifted to medium without methionine, and the kinetics of MET gene derepression was followed by Northern blot analysis. The derepression kinetics of MET3, MET16, and MET25 expression was identical in cells that expressed the Met4-Cbf1 chimera as compared with wild-type controls (Fig. 5A). Likewise, repression kinetics in response to methionine was similar in the MET4 and met4Δ:MET4-CBF1 strains (Fig. 5B). Chromatin immunoprecipitation was then used to assess the abundance of the Met4-Cbf1 chimera at individual promoters. Cross-linked chromatin was prepared from met4Δ:MET4-CBF1 cells grown in minimal B medium before and 40 min after the addition of 1 mM methionine and the Met4-Cbf1 chimera was captured with polyclonal Met4 antisera (25). Addition of methionine caused a 5- to 15-fold decrease in Met4-Cbf1 chimera occupancy at the MET3, MET16, and MET25 gene promoters, in accord with the observed methionine-mediated repression of these genes (Fig. 5C).

To further test whether repression of MET gene expression was due to the destabilization of the Met4-Cbf1 chimera, we determined the stability of an HA epitope-tagged version of Met4-Cbf1, also expressed from the chromosomal locus. The abundance of HA3-Met4-Cbf1 was rapidly diminished upon addition of methionine (Fig. 5D). That the HA3-Met4 and HA3-Met4-Cbf1 proteins were converted to similar multiple low electrophoretic mobility species and were degraded with identical kinetics argues that the Met4-Cbf1 chimera was effectively ubiquitylated by the SCFMet30 ligase. These results demonstrate that MET gene repression can occur independently of the means by which Met4 is recruited to promoter DNA and that the Met4-Cbf1 chimera is an effective substrate for the SCFMet30 ligase.

Methionine-dependent Control of Chromosome Segregation—In addition to its role in methionine biosynthesis, the Cbf1 protein binds to the centromere and participates in kinetochore complexes. Thus, in addition to methionine auxotrophy, the cbf1Δ null mutation causes a 9-fold increase in the rate of mitotic chromosome loss (21, 22, 34). As our Met4-Cbf1 chimera contains the 210–351 carboxyl-terminal fragment of Cbf1 that is sufficient to bind to the CDE1 element and to confer accurate chromosome segregation (34), we investigated the functionality of the Met4-Cbf1 chimera in centromere function. For this purpose, we examined the loss rate of a CEN-based plasmid in wild-type, cbf1Δ, and cbf1Δ cells that expressed the Met4-Cbf1 chimera from the endogenous MET4 promoter. The Met4-Cbf1 fusion protein fully suppressed the defects in plasmid segregation that are observed in the cbf1Δ null mutant such that in the absence of high extracellular methionine, the loss rate of the CEN plasmid in the met4Δ:MET4-CBF1,cbf1Δ strain was indistinguishable from wild type (Table 2). Chromatin immunoprecipitation confirmed that the Met4-Cbf1 chimera was bound to chromosomal CDE1 elements (data not shown). To assess whether the Met4-
Figure 3. Met4 degradation is proteasome dependent. A, stabilization of a GFP-Met4 fusion protein in the presence of methionine and MG132. GFP-Met4 was expressed from the GAL1 promoter (strain CYS151, ura3::pGAL1-GFP-MET4::URA3, pdr1Δ, pdr3Δ) and then repressed by addition of glucose in the presence of 1 mM L-methionine and in the absence or presence of 50, 100, or 200 µM MG132. The GFP-Met4 fluorescent signal was recorded and quantified at the indicated times using a BD Biosciences FacScaniburflow cytometer. B, cells were grown as in panel A, and the stability of GFP-Met4 was analyzed by fluorescence microscopy. C, Met4 that lacks lysine 163 is not degraded upon methionine exposure. The GFP-Met4Δ12 (strain C313, met4::TRP1, ura3::pGAL1-GFP-MET4Δ12::URA3) fusion protein was expressed in the cells from the GAL1 promoter and then repressed by the addition of glucose, either in the absence of methionine (-Met) or in the presence of 1 mM L-methionine (+Met). Fluorescence images were acquired at the indicated times. D, stabilization of the HA3-Met4Δ12 in the presence of 1 mM L-methionine. C323 (met4::TRP1, ura3::pGAL1-HA3MET4Δ12::URA3) cells were treated as in panel B. At the indicated times, proteins were extracted using the trichloroacetic acid procedure and immunoblotted with anti-HA antibody.
Cbf1 chimera was capable of responding to methionine in the context of CEN DNA, we measured CEN plasmid stability in the met4::MET4-CBF1, cbf1Δ strain in the presence of high levels of methionine. Addition of high extracellular methionine dramatically increased the rate of plasmid loss up to that observed in cbf1Δ null mutant cells (Table 2). This genetic readout for Met4-Cbf1 function parallels the methionine-induced elimination of Met4-Cbf1 and accompanying MET gene repression and thus provides additional in vivo evidence that Met4 is regulated by proteolytic mechanism in minimal medium.

**DISCUSSION**

The central role of sulfur metabolism in protein and nucleic acid biosynthesis and in overall redox status of the cell mandates an exquisite management system in which Met4 is a critical nexus. Not surprisingly then, the Met4 system is subject to multiple layers of regulation, including combinatorial transcriptional complexes that operate differentially at various MET gene promoters, auto-regulation through Met4-dependent transcription of MET30, and both degradation-dependent and independent control by SFCMet30. Here, we have delineated the intracellular signal for MET gene regulation and Met4 degradation, unequivocally demonstrated that Met4 is degraded in vivo under minimal medium conditions, and shown that Met4 can be modulated by SFCMet30 independently of local protein and DNA contexts.

Met4 Degradation Is Triggered by the Conversion of AdoMet into Cysteine—The ubiquitin-dependent degradation of Met4 was previously thought to be triggered by an increase in the intracellular AdoMet pool, as derived from the rapid conversion of extracellular methionine into AdoMet by the S-adenosylmethionine synthases encoded by the SAM1 and SAM2 genes. Thus, in cells that lack Sam1 and Sam2, methionine fails to repress MET genes, whereas high levels of extracellular AdoMet efficiently repress MET genes (35). Consistent with this view, the SAM1 and SAM2 genes were originally identified as eth2 and eth10 mutations that impaired the negative regulation of sulfur amino acid biosynthesis (36). In addition, single point mutations within MET30 allow cells to grow in the presence of a high amount of S-adenosyl-methionine, a toxic analogue of AdoMet (37). We have extended this scheme to demonstrate that Met4 degradation actually depends on the subsequent conversion of AdoMet into cysteine. Thus, when expressed from the endogenous MET4 promoter, both GFP-Met4 and H53Met4 proteins are degraded when strΔ cells are exposed to extracellular cysteine, but not to either AdoMet or methionine. The requirement for Str4 in MET gene repression by methionine and AdoMet, but not cysteine, has been noted previously (38). The here-reported cellular cysteine-triggered destabilization of both GFP-Met4 and H53Met4 expressed from the endogenous MET4 promoter exactly correlates with the results of Hansen et al. (38) demonstrating that cysteine does constitute the proximal sulfur-containing signal that mediates the repression of MET genes in cells grown in minimal medium and exposed to high extracellular methionine.

Cysteine serves as a precursor for the synthesis of glutathione and is involved in the synthesis of different vitamins, such as thiamin and biotin (39). Our results have shown that the inactivation of the GSH1 gene that codes the first enzyme of glutathione synthesis does not impair Met4 degradation as triggered by cysteine, indicating that glutathione or its derivatives are not involved in Met4 regulation, as previously suggested (40). That cysteine is the intracellular signal that controls the fate of Met4 concurs with the prominent role of Met4 both in the response to cadmium (41, 42) and in glutathione biosynthesis, which generates compensating reducing equivalents in cells exposed to severe oxidative stress (43–45). In contrast to methionine and AdoMet, cysteine and glutathione are both thiol compounds and thus directly responsive to global changes in oxidation. Intracellular cysteine is thus an ideal intermediary to control the Met4 regulon in response to the different cues of sulfur amino acid metabolism and oxidative stress defense.
Met4 Degradation Requires Nuclear Localization and Proteasome Function—Although Met4 is known to be a nuclear protein (23, 25), the sequence requirements for Met4 nuclear localization have not been determined. Our results suggest that Met4 contains two NLS regions that appear to function independently of each other. Importantly, nuclear localization of Met4 is required for its rapid elimination in response to methionine, consistent with the nuclear localization of Met30 (23, 32). Nuclear localization of the SCFCdc4 complex is required for degradation of one of its nuclear substrates, the cyclin-dependent kinase inhibitor Far1 (46). Finally, nuclear, but not cytoplasmic, elimination of the GFP-Met4Δ7 derivative in response to methionine and stabilization of GFP-Met4 by proteasome inhibitors demonstrates that Met4 is degraded in the nucleus in a conventional proteasome-dependent manner.

Context-independent Degradation of Met4—The MET gene network is controlled by several distinct transcription factors that operate in conjunction with Met4, namely Cbf1, Met28, Met31, and Met32 (16). Depending on the Met4 target gene, different complexes are formed: the Met4-Cbf1-Met28 complex assembles on the TCACGTG element present upstream of the MET16 gene, whereas the Met4-Met28-Met31/32 complexes bind to the core motif AAACTGTG present upstream of the MET3 and MET28 genes (19, 20). In the latter case, the binding of Cbf1 in the vicinity of the AAACTGTG sequences enhances the affinity of Met4-Met28-Met31/32 complexes (20). Recently, Cbf1
has been shown to interact with the chromatin-remodeling ATPase Isw1 at MET promoters and to be required for normal nucleosome positioning in promoter-proximal regions that contain TCACGTG motifs (47, 48). An important issue in the regulation of transcription by the ubiquitin system is the role of discrete contexts, both in terms of chromatin structure and protein interactions. We used a fully functional Met4-Cbf1 fusion protein to probe such effects. The kinetics of MET gene repression in response to methionine correlate precisely with promoter occupancy and abundance of the Met4-Cbf1 chimera. These results support the view that Cbf1 functions at MET gene promoters to recruit Met4 and that the synthetic Met4-Cbf1 derivative is regulated by a proteolysis in minimal medium. These experiments would appear to rule out the possibility that SCF<sup>Met30</sup>-dependent ubiquitylation of Met4 represses MET gene transcription through specific dissociation of Cbf1 from promoter DNA (24). A second important conclusion that could be derived from the properties of the Met4-Cbf1 chimera is that Met4 can be targeted by Met30 at entirely heterologous regions, as suggested by the methionine-dependent instability of CEN plasmid DNA in strains bearing Met4-Cbf1 as their only source of Cbf1 activity. Notably, centromeric DNA is transcriptionally inert, suggesting that chromatin conformation does not limit the access of SCF<sup>Met30</sup> to Met4. In addition, the methionine-dependent plasmid instability conferred by the Met4-Cbf1 fusion is consistent with the ubiquitin-mediated degradation of Met4 in minimal medium.

**Mechanism of SCF<sup>Met30</sup> Regulation**—An outstanding issue raised by these studies is the mechanism whereby intracellular cysteine levels dictate the activity of SCF<sup>Met30</sup> toward Met4. The role of cysteine in this process can in part be considered from the perspective of the evolution of the ubiquitin activation pathway. Structural studies suggest that eukaryotic ubiquitin and prokaryotic sulfur carrier ThiS (involved in thiamine biosynthesis) evolved from a common ancestor (49, 50). The first steps of ubiquitin activation are similar to the first steps of thiazole synthesis: ThiS is activated as a carboxyl-terminal acyladenylate and then converted to a thioisocarboxyylate by cysteine, precisely analogous to the activation of ubiquitin by E1 enzyme (51). It is perhaps noteworthy that apart from mutations in MET30 and core SCF subunits, no other mutations that impair SCF<sup>Met30</sup>-mediated regulation of Met4 have been identified. Thus, only a few protein components might be involved in the cysteine-dependent regulation of SCF<sup>Met30</sup> and/or Met4. As one possibility, we suggest that cysteine may directly control the activity of SCF<sup>Met30</sup> toward Met4 through a thiol-based mechanism that acts either on SCF<sup>Met30</sup> and/or on Met4. Among different hypotheses, an interesting one is that cysteine may stabilize the Met4-SCF<sup>Met30</sup> interaction by binding directly to the Met30 receptor subunit. It is worth noting that such a mechanism has been recently evidenced for the plant hormone auxin, which triggers the degradation of the Aux/IAA transcriptional repressors. Auxin was indeed demonstrated to be a ligand of the SCF<sup>Tir1</sup> ubiquitin ligase, and the binding of auxin to the receptor subunit Tir1 was shown to induce Aux/IAA degradation by promoting the Aux/IAA-SCF<sup>Tir1</sup> interaction (52). The known versatility of the thiol group in allosteric control of enzyme action (53) would certainly accommodate a mechanism in which cysteine directly regulates the activity of a specific ubiquitin ligase.

**Acknowledgments**—We thank P. Zarzov and J. De Royer for help in flow cytometry experiments.

**REFERENCES**

1. Brivanlou, A. H., and Darnell, J. E. (2002) Science 295, 69–80
2. Berger, S. L. (2002) Curr. Opin. Genet. Dev. 12, 142–148
3. Conaway, R. C., Brower, C. S., and Conaway, J. W. (2002) Science 296, 1254–1258
4. Muratani, M., and Tansley, W. (2003) Nat. Rev. Mol. Cell. Biol. 4, 192–201
5. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
6. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
7. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
8. Levinger, L., and Varshavsky, A. (1982) Cell 28, 375–385
9. Pham, A. D., and Sauver, F. (2000) Science 289, 2357–2360
10. Gonzalez, F., Delahodde, A., Kodak, T., and Johnston, S. A. (2002) Science 296, 548–550
11. Thomas, D., and Tyers, M. (2000) Curr. Biol. 10, R341-R343
12. Tansey, W. P. (2001) Genes Dev. 15, 1045–1050
13. Salghetti, S. E., Caudy, A. A., Chenoweth, J. G., and Tansey, W. P. (2001) Science 293, 1651–1653
14. Molinari, E., Gilman, M., and Natesan, S. (1999) EMBO J. 18, 6439–6447
15. Lipford, J. R., Smith, G. T., Chi, Y., and Deshaies, R. J. (2005) Cell 123, 503–512
16. Thomas, D., and Surdin-Kerjan, Y. (1997) Microbiol. Mol. Biol. Rev. 61, 503–532
17. Patton, E. E., Willems, A. R., and Tyers, M. (1998) Trends Genet. 14, 236–243
18. Willems, A. R., Schwab, M., and Tyers, M. (2004) Biochim. Biophys. Acta 1695, 133–170
19. Kuras, I., Barbey, R., and Thomas, D. (1997) EMBO J. 16, 2441–2451
20. Blaiseau, P. L., and Thomas, D. (1998) EMBO J. 17, 6327–6336
21. Baker, R. E., and Maxson, D. C. (1990) Mol. Cell. Biol. 10, 2458–2467
22. Cai, M., and Davis, R. W. (2002) Annu. Rev. Biochem. 71, 236–243
23. Rouillon, A., Barbey, R., Patton, E. E., Tyers, M., and Thomas, D. (2000) EMBO J. 19, 292–294
24. Kaiser, P., Flick, K., Wittenberg, C., and Reed, S. I. (2000) Cell 102, 303–314
25. Kuras, I., Rouillon, A., Leu, T., Barbey, R., Tyers, M., and Thomas, D. (2002) Mol. Cell 10, 69–80
26. Flick, K., Ouni, I., Wohlschlegel, J. A., Capati, C., McDonald, W. H., Yates, J. R., and Kaiser, P. (2004) Nat. Cell Biol. 6, 634–641
27. Cherest, H., and Surdin-Kerjan, Y. (1992) Genetics 130, 51–58
28. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 3019–3021

**TABLE 2**

| Strains | Growth conditions | Cell number |
|---------|-------------------|-------------|
|         | + Met | − Met | + Met | − Met | + Met | − Met | + Met | − Met | + Met | − Met |
| W303−1A + PRS313 | 348 | 352 | 388 | 277 | 378 |
| cbf1Δ + PRS313 | 307 | 291 | 257 | 247 | 277 |
| met4Δ:MET4-CBF1 | 119 | 145 | 27 | 19 | 13 |
| cbf1Δ + PRS313 | 222 | 337 | 394 | 313 | 258 |
| met4Δ:MET4-CBF1 | 195 | 296 | 338 | 298 | 227 |
| cbf1Δ + PRS313 | 13 | 12 | 14 | 5 | 12 |
| 1 mM Met | 259 | 286 | 376 | 326 | 181 | 221 | 137 | 122 |
| 0 | 30 | 52 | 62 | 58 | 61 |
Ubiquitin-mediated Degradation of Met4

Barbey, R., Baudouin-Cornu, P., Lee, T. A., Rouillon, A., Zarzov, P., Tyers, M., and Thomas, D. (2005) EMBO J. 24, 521–532

Yen, J. L., Su, N. Y., and Kaiser, P. (2005) Mol. Biol. Cell 16, 1872–1882

Jamieson, D. (2002) Nat. Genet. 31, 228–230

Pocsik, I., Prade, R. A., and Penninckx, M. J. (2004) Adv. Microb. Physiol. 49, 1–76

Lafaye, A., Junot, C., Pereira, Y., Lagriev, G., Tabet, J. C., Ezan, E., and Labarre, J. (2005) J. Biol. Chem. 280, 24723–24730

Blondel, M., Galan, J. M., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies, R. J., and Peter, M. (2000) EMBO J. 19, 6085–6097

Moreau, J. L., Lee, M., Mahachi, N., Vary, J., Mellor, J., Tsukiyama, T., and Goding, C. R. (2003) Mol. Cell. 11, 1609–1620

Kent, N. A., Eibert, S. M., and Mellor, J. (2004) J. Biol. Chem. 279, 27116–27123

Rudolph, M. J., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2001) Nature Struct. Biol. 8, 42–46

Wang, C., Xi, J., Begley, T. P., and Nicholson, L. K. (2001) Nature Struct. Biol. 8, 47–51

Furukawa, K., Mizushima, N., Noda, T., and Ohsumi, Y. (2000) J. Biol. Chem. 275, 7462–7465

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005) Nature 435, 441–445

Kiley, P. J., and Storz, G. (2004) PLoS Biol. 2, e400