The mRNA Nuclear Export Factor Hpr1 Is Regulated by Rsp5-mediated Ubiquitylation*

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Ubiquitin conjugation and in particular two distinct HECT ubiquitin ligases, Rsp5p and Tom1p, have been shown to participate in the regulation of mRNA export in Saccharomyces cerevisiae. The identification of the ubiquitin ligase substrates represents a major challenge in understanding how this modification may modulate mRNA export. Here, we identified Hpr1p, a member of the THO/TREX (transcription/export) complex that couples mRNA transcription to nuclear export as a target of the ubiquitin-proteasome pathway. Hpr1p degradation is enhanced at high temperature and appears linked to on-going RNA-polymeraseII-mediated transcription. Interestingly, the stability of the other THO complex components is not affected under these conditions indicating that Hpr1p turnover could control the formation of the THO/TREX complex and consequently mRNA export. Using in vivo and in vitro approaches we demonstrate that Rsp5p is responsible for the ubiquitylation of Hpr1p that also involves the ubiquitin-conjugating enzyme Ubc4p. Thus, Hpr1p represents the first nuclear export factor regulated by ubiquitylation, strongly suggesting that this post-translational modification participates in the coordination of transcription and mRNA export processes.

In eukaryotic cells, intracellular transport pathways are modulated as a function of differentiation, development, and cell cycle in response to a variety of stimuli. The molecular mechanisms ensuring this plasticity remain poorly characterized and probably affect both transport machineries and cargoes. Obviously, post-translational modifications and in particular ubiquitylation play a key role in control and regulation of intracellular transport. Indeed, the initial interest of ubiquitylation was focused on the role of polyubiquitin chains in targeting proteins to degradation by the 26 S proteasome. More recently, elucidating the role of mono-ubiquitylation as a sorting signal and regulator of endocytosis and endocytic trafficking led to the notion and subsequent demonstration that both mono- and polyubiquitination regulate a much wider array of cell processes, including cell cycle control, stress response, DNA repair, signaling, transcription, and gene silencing (reviewed in Ref. 1).

Ubiquitin conjugation is accomplished via a thioester cascade in which ubiquitin is first activated by a unique E1.1 In a trans-esterification reaction ubiquitin is transferred from the ubiquitin activating enzyme to an E2 ubiquitin-conjugating enzyme, which may in turn transfer the ubiquitin molecule to an E3 ubiquitin-protein ligase. Specificity of the ubiquitylation system derives from the high fidelity protein-protein interactions between E3 and the substrate (reviewed in Ref. 2). Previous studies using a yeast temperature-sensitive mutant of the ubiquitin-activating enzyme (E1) indicated that the ubiquitin pathway is involved in both poly(A)+ RNA export from the nucleus and in nuclear protein import (3). More recently, Tom1p and Rsp5p, two ubiquitin ligases (E3s) from the HECT family, have been shown to play a role in nuclear export of poly(A)+ RNA in Saccharomyces cerevisiae (4–7). The ubiquitin ligase activity of both E3s is required for its function in mRNA export, suggesting that ubiquitylation of some mRNA nuclear transport factors contribute to the regulation of this transport pathway. The identification of the ubiquitin ligase substrates represents a major challenge to understand how this modification may modulate mRNA export.

mRNA export requires that newly synthesized precursor mRNAs undergo several processing steps, which include 5′ capping, splicing, 3′-end cleavage, and polyadenylation. The different steps leading to mRNP formation are linked to each other and often mediated by interactions with the RNA polymerase II transcription machinery (8). All these functional couplings may be part of quality control mechanisms ensuring that only fully mature transcripts reach the cytoplasm (9). A considerable number of proteins have been implicated in the export of mRNA, including Mex67p (TAP in metazoan). Mex67p (TAP) acts as a transporter that interacts with export competent mRNPs and mediates its docking and translocation through the nuclear pore complexes. As Mex67p exhibits low affinity for RNA, its interaction with mRNAs is mediated by adaptor proteins like Yra1p, an essential hnRNP-like protein of the REF family (10–14). Recruitment of Yra1p and its partner Sub2p, an ATPase/RNA helicase essential for mRNA export, occurs co-transcriptionally and is splicing-independent (15–18). Recent findings established a more direct link between Yra1p, Sub2p, and the transcription machinery, since both

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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; HA, hemagglutinin; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; GFP, green fluorescent protein; CTD, C-terminal domain; RNP, ribonucleoprotein; hnRNP, heterogenous RNP; mRNP, messenger RNP.
proteins are intimately associated to components of the THO complex, which has been implicated in transcription elongation, transcription-dependent recombination, and mRNA export (18–20). Genomic deletion of genes encoding THO complex components (HPR1, THO2, MPT1, THP2) is viable at 23 °C but leads to poly(A) RNA export defect and cell death at 37 °C indicating that this complex is dispensable at 23 °C but essential at higher temperature (20). THO, Sub2p, and Yra1p are recruited coincidentally to an active gene during transcription elongation. Sub2p can be recruited to the nascent transcript by virtue of a direct interaction with Hpr1p and may in turn facilitate the binding of Yra1p to the mRNA (18). It was proposed that the binding of Mex67p to Yra1p is at higher temperature (20) indicating that this complex is essential at 30 or 37 °C. Second, besides its catalytic domain, the role of Rsp5p on nuclear transport is mediated by its second and third WW repeats (6) thought to interact with Rsp5p substrates through a PY motif (22–25). Here we show that the THO complex component Hpr1p is ubiquitylated and degraded both in vitro and in vivo by Rsp5p in conjunction with the E1 and Ubc4p as an E2.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth**—The W303, YPS1675, DF5, cim3.1, Δerg, Δrps5, Δtom1, Sub2, and rpb1Δ strains were described previously (5, 18, 26–31). HPR1, HPR1Δ88, and THP2 were genomically tagged with an HA epitope in a W303 background or cim3.1 cells as described previously (32). Δrps5 cells were grown in the presence of oleic acid as described (27, 6). CIM3 and cim3.1 strains transformed with the HisΔ ubiquitin vector were grown on selective media supplemented with 0.1 g/ml of CuSO4, 50 mM MgOAc, 2.5 mM tris(2-carboxyethyl)phosphate, 500 mM NaCl, and 50% glycerol, 1 μg of ubiquitin, 0.16 μg of E1, 3.8 μg of Ubc4 E2, 1.2 μg of Rep5 E3, 8 pmol of GST-tagged substrate, and 3.3 mM ATP when indicated. Reactions were allowed to proceed for 4 h at room temperature and stopped by boiling in 5 μl of SDS-PAGE sample buffer containing 3 mM urea.

**RESULTS AND DISCUSSION**

**Hpr1p Degradation Is Temperature-dependent**—It has been clearly shown that Rsp5p- and Tom1-dependent ubiquitylation, but also expression of the THO complex components, are dispensable for mRNA export at 23 °C but essential at 37 °C (6, 17, 20). To determine whether the THO complex, or at least one of its components, could be a substrate for the Rsp5p or Tom1p ubiquitin ligases resulting in the regulatory function of these enzymes in mRNA export, stability of the THO complex proteins was first analyzed in different conditions. The stability of THO components was measured at both 23 °C and 37 °C by cell treatment with the protein synthesis inhibitor cycloheximide (+CX) and analysis of expression by Western blotting using specific antibodies (Fig. 1A). Using this approach, genomically HA-tagged Hpr1p (Hpr1-HA) or untagged Hpr1p (data not shown) exhibited a half-life of about 30 min at 23 °C that was dramatically reduced to below 15 min at 37 °C (Fig. 1A). Hpr1p synthesis did not appear to fully compensate for this accelerated degradation, as indicated by the progressive decrease in the cellular content of Hpr1p upon a shift to 37 °C (+CX). Increased instability of Hpr1-HA at 37 °C was confirmed by pulse-chase experiment followed by immunoprecipitation of Hpr1-HA (Fig. 1B). In contrast to Hpr1p, the THO components Mtr1p and genomically HA-tagged Thp2p, as well as the export receptor Mex67p, were rather stable at both 23 and 37 °C indicating that this highly active metabolism specifically concerns Hpr1p (Fig. 1A). Because of its low abundance (10-fold less than Mtr1p), the stability of Tho2p was not analyzed in this study. Interestingly, deletion of the C-terminal 88 amino-acids that preserved the Hpr1p nuclear localization signal prevented degradation of Hpr1Δ88 at 23 °C and clearly slowed down its turnover at 37 °C (Fig. 1, A and B) indicating that the C-terminal domain of Hpr1p likely contains the target amino acid residues for accelerated degradation. Deletion of this region does not strongly affect viability at 37 °C suggesting that this truncation does not prevent formation of the THO complex.

**Degradation of Hpr1p Depends on Active RNA Polymerase II-mediated Transcription**—Proteins of the THO complex co-purify with the DEAD-box RNA helicase Sub2p and the mRNA export adaptor Yra1p in a complex called TREX, proposed to couple transcription and nuclear export (20). Studies on the THO components of TREX indicate that the recruitment of Hpr1p occurs during transcription elongation (18, 20, 38). We thus analyzed the requirement of the transcription process in the ubiquitin-proteasome-mediated degradation of Hpr1p. For this purpose, cells were treated with the transcription inhibitor thiolutin for 15 min prior to shifting the cells to 37 °C. As shown on Fig. 1C, thiolutin led to a clear stabilization of Hpr1p at 37 °C. To confirm this result, Hpr1p expression was also analyzed in cells containing a thermosensitive mutation for Rpb1, the large RNA polymerase II subunit. We found that...
with a 20 degradation of Hpr1p at 37 °C was prevented in cells treated in inhibitors (29). Results shown in Fig. 2 analyzed in sensitivity of Hpr1p expression to proteasome inhibitor was determined in wild-type and the THO Complex Factor Hpr1p—

Hpr1p turnover. These results illustrate that on-going transcription is required for the Rsp5p ubiquitin ligase.

Ubiquitin- and Proteasome-dependent Degradation of Hpr1p—To determine whether degradation of Hpr1p was dependent on the proteasome activity, the sensitivity of Hpr1p expression to proteasome inhibitor was analyzed in Δerg cells, which enable the uptake of peptide inhibitors (29). Results shown in Fig. 2A clearly indicated that degradation of Hpr1p at 37 °C was prevented in cells treated with a 20 μM concentration of the proteasome inhibitor MG132. Consistent with these data, Hpr1p levels were stabilized at 37 °C in cim3.1 thermosensitive mutants, impaired in proteasome activity (28), compared with the corresponding wild-type CIM3 strain (Fig. 2B). Since recognition and degradation by the 26 S proteasome requires polyubiquitination of the substate, modification of Hpr1p was observed in wild-type and cim3.1 cells overexpressing His-tagged versions of ubiquitin from a copper-inducible vector. Cells were maintained at 23 °C or shifted to 37 °C for 4 h. His6-ubiquitin-conjugated proteins from the cell lysates were purified on nickel columns and analyzed by Western blotting using an anti-Hpr1p antibody. With this approach, polyubiquitin-conjugated species of Hpr1p could be detected in extracts of cim3.1 cells expressing His6-tagged ubiquitin and grown at the restrictive temperature. In contrast, wild-type cells and mutant cells grown at the permissive temperature or transformed with a control vector presented a weak or no detection of ubiquitylated Hpr1p (Fig. 2C). Altogether, these results clearly indicate that Hpr1p is polyubiquitylated and actively metabolized by the proteasome-mediated degradation pathway.

Hpr1p Is a Substrate for the Rsp5p Ubiquitin Ligase—The catalytic activity of the ubiquitin ligase Rsp5p and Tom1p has been shown to regulate efficiency of mRNA nuclear export (5–7). To determine whether one of these ligases is responsible for Hpr1p ubiquitylation, Hpr1p degradation was analyzed in rsp5Δ or tom1Δ strains. Although disruption of the RSP5 gene is lethal, cell viability at 23 °C, but not at 37 °C, can be rescued by adding oleic acid to the growth medium (27). Indeed the processing-mediated activation of the transcription factor Sp123p by Rsp5p is required for the synthesis of Ole1p, an endoplasmic reticulum-bound enzyme essential for lipid and membrane synthesis which catalyzes the desaturation of C16 and C18 fatty acids and formation of palmitoleic and oleic acids. Disruption of TOM1 or RSP5 did not affect the steady-state expression of Hpr1p at 23 °C. In contrast, Hpr1p degradation was completely abolished at 37 °C in a rsp5Δ strain and not in a tom1Δ strain (Fig. 3A), suggesting that ubiquitylation of Hpr1p is likely to be mediated by the Rsp5p ubiquitin ligase.
Ubiquitylation of Hpr1

Covalent attachment of ubiquitin molecules is mediated through a thiol ester cascade of reactions catalyzed by a unique ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). Both ubiquitin-conjugating enzymes Ubc4p and Ubc5p have been described as partners for the Rsp5p ligase (39). To test whether one of these enzymes is required for the ubiquitylation and subsequent degradation of Hpr1p, Hpr1p expression was analyzed at 23 °C or after a 1-h shift at 37 °C in \textit{ubc4}\textsubscript{Δ}, \textit{ubc5}\textsubscript{Δ}, \textit{ubc4}\textsubscript{Δ}\textit{ubc5}\textsubscript{Δ} mutant cells. As a control, we also used cells disrupted for \textit{UBC6} and/or \textit{UBC7} genes encoding for ubiquitin-conjugating enzyme involved in particular in the degradation of membrane proteins (31). As shown in Fig. 3C, temperature-induced Hpr1p degradation was not affected in \textit{ubc5}\textsubscript{Δ}, \textit{ubc6}\textsubscript{Δ}, \textit{ubc7}\textsubscript{Δ} cells but was prevented in \textit{ubc4}\textsubscript{Δ} cells with a more pronounced effect in the absence of both Ubc4p and Ubc5p enzymes. Although Ubc4p and Ubc5p have been shown to be highly similar and functionally redundant, \textit{UBC4} is mainly transcribed during the exponential phase of growth and stimulated by heat shock, whereas transcription of \textit{UBC5} occurs during the stationary phase (30). This differential pattern of expression could explain the predominant but not exclusive role of Ubc4p in the ubiquitylation of Hpr1p at 37 °C. Altogether these results show that Ubc4p, and probably Ubc5p, and Rsp5p are required for the ubiquitylation of Hpr1p in \textit{vivo}.

\textit{E1}, Ubc4p, and Rsp5p Are Necessary and Sufficient to Ubiquitylate Hpr1p in \textit{Vitro}—To test whether \textit{E1}, Ubc4p, and Rsp5p are not only required but also sufficient to promote polyubiquitylation of Hpr1p, \textit{in vitro} ubiquitin conjugation reactions with recombinant GST-Hpr1 were carried out in the presence of recombinant purified enzymes. GST fused to the C-terminal domain (CTD) of RNA polymerase II large subunit was used as a control (40). As shown in Fig. 3D, a marked increase in the amount of high molecular weight conjugates and a corresponding decrease in the level of unmodified GST-Hpr1 and GST-CTD was observed in reactions performed in the presence of ATP. No modification could be detected when the assay was performed in the absence of one of the enzymes or in the presence of Ubc1p as an alternative E2 (not shown). In addition, modification of GST-Hpr1 appeared dependent on the concentration of the substrate and reached a plateau with 500 nM GST-Hpr1 (Fig. 3D and data not shown). Although we cannot formally exclude that binding of Rsp5p to Hpr1p can be regulated by an additional partner \textit{in vivo}, results presented here clearly demonstrate that interaction between this enzyme-substrate complex does not require any adaptor \textit{in vitro} and might involve the recognition of the LPxY motif of Hpr1p.

To further investigate whether Hpr1p specifically interacts with Rsp5p, we generated a GST fusion protein expressing the WW2/WW3 domains (amino acids 281–429) of this E3 ligase, a region previously implicated in the nuclear export of poly (A) RNA (6). A lysate from cells expressing an HA-tagged version of the Rsp5p partner Bul1p or Thp2p or GFP-tagged Mft1p. Untagged Mft1p and Mex67p were analyzed from cells expressing Hpr1-HA. Beads were washed, and bound material was analyzed by Western blotting. 0.5% of the input was loaded in lane 1 (extracts). C, indicated cell strains were grown at 23 °C or shifted to 37 °C during the indicated periods of time (minutes). D, GST or GST-WW2/WW3 Rsp5 recombinant proteins on glutathione-Sepharose beads were incubated with extracts from cells expressing HA-tagged versions of Bul1, Hpr1p, or Thp2p or GFP-tagged Mft1p. Untagged Mft1p and Mex67p were analyzed from cells expressing Hpr1-HA. Beads were washed, and bound material was analyzed by Western blotting. 0.5% of the input was loaded in lane 1 (extracts). C, indicated cell strains were grown at 23 °C or shifted to 37 °C. D, ubiquitylation of indicated amount of GST-Hpr1 or GST-CTD was realized in the presence of recombinant E1, Ubc4, and Rsp5. ATP was either omitted or added last to minimize auto-catalytic ubiquitylation reactions by the ubiquitylation enzymes. Cell extracts or in vitro reactions were analyzed by Western-blot with indicated antibodies.

\*B. Kus and A. M. Edwards, personal communication.
over of Hpr1p depends on an active on-going transcription, we
would like to propose that dissociation of the THO complex may be
coupled to the transcription process and possibly involved in
mature mRNP release. Defects in Rsp5p ligase activity or in
Hpr1p expression would thus have direct consequences on
mRNA export as it has been previously observed (6, 7, 20).
Evidence has been accumulating over the last few years sug-

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