In this study we demonstrate that the Deg1 degradation signal of the transcriptional repressor Mata2 confers compartment-specific turnover to a reporter protein. Rapid degradation of a Deg1-containing fusion protein is observed only when the reporter is efficiently imported into the nucleus. In contrast, a reporter that is constantly exported from the nucleus exhibits an extended half-life. Furthermore, nuclear import functions are crucial for both Deg1-induced degradation as well as for the turnover of the endogenous Mata2 protein. The conjugation of ubiquitin to a Deg1-containing reporter protein is abrogated in mutants affected in nuclear import. Obviously, the Deg1 signal initiates rapid proteolysis within the nucleoplasm, whereas in the cytosol it mediates turnover via a slower pathway. In both pathways the ubiquitin-conjugating enzymes Ubc6p/Ubc7p play a pivotal role. These observations imply that both the cellular targeting of a substrate and the compartment-specific activity of components of the ubiquitin-proteasome system define the half-life of naturally short-lived proteins.

Rapid inactivation of regulatory factors is an essential step in many central biological processes including cell cycle control and signal transduction (1, 2). Down-regulation of these factors often occurs by specific proteolysis mediated by the ubiquitin system. In a brief and simplified view, the conjugation of substrates with ubiquitin involves the successive action of three classes of enzymes: The E13, ubiquitin-activating enzyme, E2s or ubiquitin-conjugating enzymes (Ubc), and occasionally E3s or ubiquitin-protein ligases. In most cases described in vivo the ubiquitin-conjugating enzymes Ubc6p/Ubc7p play a pivotal role. These observations imply that both the cellular targeting of a substrate and the compartment-specific activity of components of the ubiquitin-proteasome system define the half-life of naturally short-lived proteins.

One of the first and so far best characterized degradation signals is found in the transcriptional repressor Mata2 of Saccharomyces cerevisiae and was termed Deg1 (6). This signal comprises the first 67 amino acids of the Mata2 protein, and its recognition involves the E2 enzymes Ubc6p and Ubc7p (7). It has been postulated that a hydrophobic surface of an amphipathic helix present in Deg1 (amino acids 14–32) is the major structural feature of this signal (8). It is noteworthy that the Deg1 signal also contains a putative nuclear localization signal (9).

Recently, it became evident that the components of the ubiquitin system are not evenly distributed throughout the cell. A prominent example for the compartmentalization of the system is endoplasmic reticulum-associated protein degradation (ERAD) (10, 11). Interestingly, Ubc6p and Ubc7p and its membrane receptor Cue1p are also crucial components of ERAD (12–15). Another proteolytic event, which seems to be restricted to a specific compartment, is the degradation of mitotic regulators. In yeast, the subunits of the involved E3 complex termed anaphase-promoting complex or cyclosome are mainly found in the nucleoplasm (16). Furthermore, it has been reported that the 26 S proteasome is concentrated in and around the nucleus at least in yeast cells (17). However, it remains to be clarified in which compartment anaphase-promoting complex-mediated proteolysis actually takes place.

Here, we demonstrate for the first time that rapid and specific proteolysis of a short-lived protein is restricted to a certain cellular compartment. Although the full-length Mata2 is an exclusively nuclear protein that is extremely short-lived, the isolated Deg1 signal confers no defined localization to a reporter protein and is degraded with slower kinetics. When a classical SV40 type NLS (18) (termed cNLS) is fused to Deg1 its turnover follows the criteria of Mata2 proteolysis. Thus, a degradation signal has to be combined with localization signals directing it to its native compartment.

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Methods—Yeast-rich and minimal media were prepared as described, and standard genetic methods were used (19). The Escherichia coli strain used was XL1 Blue, and standard techniques were employed for recombinant DNA work (19).

Plasmid Construction—All fusion proteins used in this study were under control of the copper-inducible CUP1 promoter and expressed from an ARS/CEN plasmid. To increase the pace of maturation as well as fluorescence, yeGFP was used. This version of GFP is adapted to the codon usage of S. cerevisiae and, in addition, carries the mutations S65G and S72A (20). The polymerase chain reaction (PCR) was used to generate a tandem cassette of yeGFP, which was ligated to a CUC1 terminator sequence and then subcloned into pRS416. The CUP1 promoter sequence and the wild-type MATa2 sequence and its Deg1-encoding region, corresponding to the N-terminal 67 amino acids (6, 7), were amplified separately by PCR using Pfu polymerase (Stratagene, La Jolla, CA) from yeast genomic DNA. The PCR products were digested, ligated, and subsequently re-amplified by a second PCR. The
resulting DNA fusions Cup1-Mat2p and Cup1-Deg1 were digested and then inserted in-frame N-terminal to the GFP tandem into the pRS416-GFP-CYC1 plasmid to generate Mat2-GFP (pUL32) and Deg1-GFP (pUL28) expression constructs. The structure of the protein expressed from pUL32 is as follows: Mat2p (amino acids Met-1 to Glu-210)-PGIP-yeGFP (Met-1 to Lys-238)-EKKIP-yeGFP2 (Met-1 to Lys-238). The chimaeric protein expressed from pUL28 consists of: Deg1 (Met-1 to Ile-67 of Mat2p)-TEGIP-yeGFP1 (Met-1 to Lys-238)-EKKIP-yeGFP2 (Met-1 to Lys-238). The hybrids Deg1-NES-GFP (pUL29) and Deg1-P12-GFP (pUL42) were constructed by cloning the desired oligonucleotides (21) in-frame between the Deg1 sequence and the C-terminal yeast GFP tandem into the plasmid pUL28. The plasmids pUL46 and pUL47 expressing the Deg1-NLS-GFP and Deg1-K128T-GFP fusion proteins, respectively, were generated by cloning the oligonucleotides 16171 (5′-AATCCACTCTACCCACCCCAAGAAGAAAGAAGGTTGAGACCCAAAAGGG) and 16172 (5′-GATCCCCCTTTGGGTCTTCAACCTTTCTTCTTCTTGTTGTTAGTGGG) for the SV40 NLS and 16173 (5′-AATCCACTCTACCCACCCCAAGAAGAAAGAAGGTTGAGACCCAAAAGGG) and 16174 (5′-GATCCCCCTTTGGGTCTTCAACCTTTCTTCTTCTTCTTGTTGTTAGTGGG) for the nonfunctional SV40 K128 TNLS in-frame between Deg1 and the two C-terminal yeast GFP moieties of pUL28. PCR mutagenesis of the plasmids pUL28 and pUL46 was used to introduce the amino acid substitutions K3E, P5A, and K7E into the fusion protein. The resulting Deg1* constructs were named pUL30 (Deg1*-GFP) and pUL34 (Deg1*-NLS-GFP), respectively. All constructs were sequenced, and SDS-polyacrylamide gel electrophoresis and Western blotting verified the size of the expressed GFP fusion proteins. Detection with a polyclonal antibody specific for fusion proteins of the Deg1 signal with respect to its nuclear cytosolic partitioning. For this purpose, we constructed fusion proteins of the Deg1 signal fused to GFP exhibits the same nuclear localization as the model substrate to study Deg1-dependent proteolysis. We used a modified form of GFP (yeGFP) (22), which folds evenly distributed throughout the cell (data not shown). Pas- sive diffusion of the reporter proteins through the nuclear pore was prevented using a tandem array of two yeGFP moieties. The localization of GFP fusion proteins was monitored with a fluorescence microscope and, protein half-lives were determined in pulse-chase experiments followed by quantification. In no instance, breakdown intermediates have been observed. It is of note that the rate of synthesis as well as the degrada- tion kinetics of the Deg1-GFP fusion proteins were similar to those of a previously described Deg1*-β-galactosidase reporter, both in wild-type and Δube6 cells (data not shown). Thus, the generated Deg1-GFP reporter was considered to be a suitable model substrate to study Deg1-dependent proteolysis.

First we tested whether the isolated Deg1 degradation signal fused to GFP exhibits the same nuclear localization as the

### Results

We wanted to investigate proteolysis initiated by the Deg1 signal with respect to its nuclear cytosolic partitioning. For this purpose, we constructed fusion proteins of the Deg1 signal with GFP. We used a modified form of GFP (yeGFP) (22), which folds rapidly in yeast and thus allows the investigation of short-lived fusion proteins. On its own, yeGFP is a stable protein, which is evenly distributed throughout the cell (data not shown). Passive diffusion of the reporter proteins through the nuclear pore was prevented using a tandem array of two yeGFP moieties.

The localization of GFP fusion proteins was monitored with a fluorescence microscope, and protein half-lives were determined in pulse-chase experiments followed by quantification. In no instance, breakdown intermediates have been observed. It is of note that the rate of synthesis as well as the degradation kinetics of the Deg1-GFP fusion proteins were similar to those of a previously described Deg1*-β-galactosidase reporter, both in wild-type and Δube6 cells (data not shown). Thus, the generated Deg1-GFP reporter was considered to be a suitable model substrate to study Deg1-dependent proteolysis.

First we tested whether the isolated Deg1 degradation signal fused to GFP exhibits the same nuclear localization as the
native Matα2 protein. We observed that the endogenous NLS of Deg1 is not sufficient to direct the fusion protein exclusively to the nucleus, which is in agreement with results reported earlier (9). In contrast to a GFP-tagged version of the full-length transcription factor Matα2 (Fig. 1A) and to a cNLS-GFP reporter protein (data not shown), both of which are nuclear proteins, a large portion of Deg1-GFP was found in the cytoplasm. Therefore, we examined whether the half-life of Deg1-GFP would be influenced by this altered localization. To this end, we inserted the cNLS between Deg1 and the GFP segment (Deg1-cNLS-GFP). When expressed in wild-type cells this fusion protein was found exclusively in the nucleus (Fig. 1A).

Intriguingly, its turnover was strikingly faster than that observed for Deg1-GFP (Fig. 1B). To exclude that the inserted protein was localized to the nucleus.

**Fig. 1. Deg1-containing fusion proteins display rapid turnover when localized to the nucleus.** A, the subcellular localization of various Deg1-containing fusion proteins was analyzed in wild-type cells grown at 30 °C. The N-terminal endogenous NLS reported for Deg1 (Hall et al. (9)) is obviously not sufficient to localize Deg1-GFP exclusively to the nucleus. Instead, a large portion of the fusion protein was found in the cytoplasm. In addition, cells often exhibit one or more intense spots of fluorescence against a diffuse fluorescent background. In clear contrast to this, both GFP-tagged Matα2 (Matα2-GFP) and the fusion protein with the inserted classical SV40 NLS between Deg1 and the GFP segment (Deg1-cNLS-GFP) are exclusively localized to the nucleus. Fusion proteins containing a nonfunctional SV40 NLS (Deg1-K128T-GFP) are distributed like Deg1-GFP and also exhibit spots of intense fluorescence. B, enhanced turnover of nuclear targeted Deg1-cNLS-GFP. Pulse-chase analysis (representative sample) and plot of Deg1-GFP, Deg1-cNLS-GFP, and Deg1-K128T-GFP degradation in haploid wild-type cells are shown. The chart and the calculated half-life represent averaged data of five (Deg1-GFP, Deg1-cNLS-GFP) and three (Deg1-K128T-GFP) individual pulse-chase experiments at 30 °C, respectively.
FIG. 2. Rapid Deg1-induced turnover depends on nuclear import functions. 

A, blockage of nuclear import stabilizes Deg1-cNLS-GFP. Pulse-chase analysis of Deg1-cNLS-GFP at 37 °C comparing wild type with \textit{nup49–313} and \textit{nsp1tsL640S} mutant cells. Prior to pulse labeling cells were shifted for 5 h to the nonpermissive temperature of 37 °C. The chart and the calculated half-life show averaged pulse-chase data of three individual experiments.

B, ubiquitination of Deg1-cNLS-GFP is absent from a yeast strain defective in nuclear import. Wild-type and mutant strains expressing GFP fusion protein and Myc epitope-tagged ubiquitin as indicated were lysed. GFP was immunoprecipitated with a specific
cNLS generally destabilizes Deg1-GFP, we replaced this cNLS by a mutant one (K128T) (22). Deg1-GFP containing this non-functional cNLS is neither exclusively localized to the nucleus (Fig. 1A) nor rapidly degraded (Fig. 1B). Taken together, these data support the idea that nuclear import of a proteolytic substrate may be a prerequisite for efficient degradation via the ubiquitin-proteasome pathway.

Next, we monitored the influence of a block in nuclear import on proteolysis of Deg1-cNLS-GFP. We used two temperature-sensitive yeast mutants, nsp1tsL640S (23, 24) and nup49–313 (25, 26), in which nuclear import is affected because of mutations in genes encoding components of the nuclear pore complex. Upon shift to the nonpermissive temperature, Deg1-cNLS-GFP was redistributed mainly to the nuclear periphery both in nsp1tsL640S and nup49–313 cells (data not shown), and its turnover was reduced to the level of Deg1-GFP (compare Fig. 2A with Fig. 1B). Ubiquitin-proteasome-dependent degradation of an ERAD model substrate (CPY⁺) (10, 11) occurred normally in these mutants (data not shown) indicating that nuclear import did not affect general activities of the proteolytic system. Furthermore, we used the srp1–49 mutant that specifically prevents the nuclear import of proteins carrying the classical SV40 NLS (27). In this mutant Deg1-cNLS-GFP was relocalized to the cytosol (data not shown), and its turnover was slowed down dramatically (Fig. 2C). In contrast, proteolysis of Deg1-GFP, which does not contain the cNLS, proceeded as in wild-type cells (Fig. 2D).

To provide further evidence for a specific degradation pathway of the nucleus we monitored the ubiquitination of Deg1-cNLS-GFP. For this purpose, we expressed an epitope-tagged version of ubiquitin (28) in wild-type, nup49–313, and pre1–1 (29) cells, the latter of which is affected in proteasomal activities. In the samples prepared from wild-type or the pre1–1 strain we observed ubiquitinated species of Deg1-cNLS-GFP. Surprisingly, we failed to detect such species in nup49–313 cells (Fig. 2B), indicating that nucleus-specific ubiquitin conjugation pathways are important for the proteolysis of substrates localized to this compartment.

So far, all experiments described have been performed with fusion proteins containing the Deg1 signal. To test the physiological relevance of our observations for the degradation of the endogenous Mato2 protein we monitored the proteolysis of this transcription factor in nsp1tsL640S and nup49–313 strains. In both mutants, a clear delay in Mato2 turnover was observed (Fig. 2E). The degree of stabilization was comparable with that of a Δubc4Δubc6 mutant in our genetic background (data not shown), indicating that nuclear import defects delay a major proteolytic pathway of the Mato2 protein. In addition, the data are consistent with those obtained with the Deg1-cNLS-GFP reporter protein and support the functional significance of a proteolytic pathway specific for Mato2 in the nucleus.

All experiments described above point to the fact that nuclear import might be a prerequisite for rapid proteolysis initiated by Deg1. Because Deg1 contains a putative NLS at the very N terminus, fusion proteins of it are, although inefficiently, imported into the nucleus. Therefore, their half-life should increase when import into the nucleus is prevented. To investigate this issue, we mutated the putative endogenous NLS present in the Deg1 signal. In analogy to reported mutations in the cNLS (22) we replaced lysine 3 and 7 with alanine. In addition, proline 5 was converted into alanine. In wild-type cells, the turnover of this Deg1*-GFP fusion protein was slower than that of Deg1-GFP, although an altered localization of Deg1*-GFP was not observed (data not shown). Transport of Deg1*-GFP into the nucleus by an insertion of a cNLS (Deg1*-cNLS-GFP) led to accelerated proteolysis. However, turnover of this protein was significantly slower than that of Deg1-cNLS-GFP (data not shown). Obviously, the introduced mutations in the N terminus of Deg1 seem to interfere with proper recognition of the degradation signal or with its ubiquitination.

Because of the physical linkage of the Deg1 signal and its putative endogenous NLS, we performed an alternative experiment. We inserted a leucine-rich nuclear export signal (NES) specific for the Xpo1 pathway (21) into Deg1-GFP to redirect the imported molecules into the cytosol. As a control, we used Deg1-GFP containing a nonfunctional NES (P12NES) (30). Insertion of the NES resulted in nuclear exclusion of the reporter protein whereas P12NES had no influence on the nuclear cytosolic partitioning (Fig. 3A). The half-life of the NES-containing fusion was slightly but significantly increased, whereas the turnover of the reporter carrying the nonfunctional NES remained unchanged (Fig. 3B). Taken together these results are consistent with the assumption that at least a small portion of Deg1-GFP is subjected to rapid proteolysis upon entering the nucleus. We therefore proposed the existence of a rapid nuclear and a slow cytosolic degradation pathway specific for the Deg1 signal. To characterize both pathways in more detail we monitored proteolysis of our reporter proteins in various mutants in components of the ubiquitin system. Degradation of both Deg1-cNLS-GFP and Deg1-NES-GFP is dependent on Ubc6p and Ubc7p (Fig. 4, A and B) but independent of Ubc4p (data not shown). However, the degree of stabilization of the two reporters was different; the fusion protein, which was found mainly in the cytosol, was largely stabilized in the absence of Ubc6p or Ubc7p (roughly 3–7-fold). In contrast, the stabilization was weaker when the reporter protein was efficiently transported into the nucleus (2-fold). Similarly, we found that Deg1-NES-GFP was strongly stabilized in Δcue1 cells (4–5-fold), whereas Deg1-cNLS-GFP had a half-life comparable with that of wild-type cells (Fig. 4, C and D). Thus, the influence of Ubc6p and Ubc7p on the rapid and nucleus-specific Deg1 degradation pathway was much smaller than on the slow cytosolic one. In the case of Cue1p, an influence on the nuclear pathway was hardly measurable.

**DISCUSSION**

The aim of this study was to clarify whether fast and efficient proteolysis of a short-lived protein not only depends on the presence of a degradation signal but also requires a specific localization of the substrate. To investigate this issue, we constructed fusion proteins consisting of GFP and the degradation signal contained in Mato2 termed Deg1. Additional cNLS (classical SV40 type) or leucine-rich NES (specific for the Xpo1 pathway) were introduced to direct them either to the nucleus or to the cytosol. This experimental approach allowed the spe-
specific localization of the substrate in wild-type cells without affecting nucleocytoplasmic transport in general, which could result in unspecific secondary effects on the ubiquitin-proteasome pathway. The generated fusion proteins behaved differently; Deg1-cNLS-GFP and Deg1-NES-GFP were found exclusively in the nucleus and cytosol, respectively. Intriguingly, the cNLS-containing fusion protein is turned over rapidly whereas the NES-containing reporter exhibits an increased half-life compared with Deg1-GFP. Because increased turnover of the substrate correlated with its nuclear localization whereas slow proteolysis correlated with cytosolic localization, we conclude that the Deg1 degradation signal mediates fast proteolysis only in the nucleus. Further support for this idea is provided by the observation that proteolysis of the cNLS-containing fusion protein as well as its ubiquitination is strongly impaired in mutants defective in nuclear import functions. We consider it
unlikely that ubiquitin-mediated proteolysis in general is slowed down in these mutants because the degradation of a characteristic ERAD substrate (CPY*) proceeded as expected. The conclusions drawn from the experiments with the Deg1 signal alone are of physiological relevance for the degradation of the Mata2 transcriptional repressor because of the following reasons. First, turnover of the endogenous Mata2 was also delayed in nuclear import mutants. Second, the influence of a 

\[ \Delta \text{cue1} \] mutation on Mata2 degradation was significantly lower than that of a \[ \Delta \text{ubc6} \] or \[ \Delta \text{ubc7} \] allele. Third, a rapidly exported Mata2-NES-GFP fusion protein displayed a drastically prolonged half-life compared with a similar fusion protein lacking reasons. First, turnover of the endogenous Mata2 was also delayed in nuclear import mutants. Second, the influence of a 

\[ \Delta \text{cue1} \] mutation on Mata2 degradation was significantly lower than that of a \[ \Delta \text{ubc6} \] or \[ \Delta \text{ubc7} \] allele. Third, a rapidly exported Mata2-NES-GFP fusion protein displayed a drastically prolonged half-life compared with a similar fusion protein lacking...
the NES (data not shown). The latter results and those on the Deg1-NES-GFP fusion protein strongly support the hypothesis that the half-life of Mata2 is a function of the time the protein remains in the nucleus.

In addition, we observed qualitative differences between cytosolic and nuclear turnover of Deg1-containing fusion proteins. The stabilization of Deg1 caused by a lack of Ubc6p/Ubc7p is stronger in the cytosol than in the nucleus. Similarly, the influence of Cue1p on Deg1-induced proteolysis seems to be minimal in the nucleus, whereas in the cytosol Cue1p plays a crucial role. The slight stabilization of Deg1-cNLS-GFP observed in Δcue1 cells is most likely due to the fact that the level of Ubc7p is altered in these cells (15). The slow turnover of Deg1-NES-GFP in the cytosol most likely takes place at the endoplasmic reticulum membrane because it was reported that degradation of a Deg1-β-galactosidase fusion protein relied completely on the function of Cue1p and hence on the anchoring of Ubc7 to the endoplasmic reticulum membrane (15). However, Deg1-β-galactosidase degradation was independent of Hrd1p/Der3p and Der1p (data not shown) and thus differed from that of a typical ERAD substrate like CPY*. These differences between turnover in the cytosol and in the nucleus are most likely of physiological importance. If the rapid Mata2 degradative system (t1/2 = 4 min) (6) would also be active in the cytosol many of the newly synthesized molecules would be degraded before they reach their place of action.

Taken together our data demonstrate that the half-life of a protein is determined by specific degradation and localization signals. The dependence on localization signals is probably caused by the restriction of components of the ubiquitin system to certain compartments. For example, a Deg1-recognizing E3 enzyme may be exclusively found in the nucleus and allows rapid turnover solely in this compartment. Without this specificity-conferring component (in the cytosol) Ubc6p/Ubc7p are able to recognize the signal but with lower efficiency. Our data imply that the nucleus is a crucial compartment for Mata2 proteolysis. Other examples have been presented in which nuclear export is also a specific prerequisite for rapid proteolysis (31–33). In cell biological terms, nucleocytoplasmic transport might represent a mechanism to regulate a protein’s half-life. By transporting a protein into a different compartment, the turnover might be up- or down-regulated probably because certain components of the ubiquitination cascades are restricted to certain areas within an eukaryotic cell.

Acknowledgments—Gene constructs, yeast strains, and antibodies were kindly supplied by A. J. P. Brown (yeGFP), C. Enenkel (srp1–49), E. Hert (nat49–313, nat14–640S), D. Wolf (pre1–1), D. Finley (Myc-tagged ubiquitin construct yEP105), and M. Hochstrasser (anti-Mata2 antisera). For helpful discussions and critical reading of the manuscript we are grateful to Ernst Jarosch, Ulrike Kutay, and Katrin Stade.

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