Cic1, an adaptor protein specifically linking the 26S proteasome to its substrate, the SCF component Cdc4

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In eukaryotes, the ubiquitin–proteasome system plays a major role in selective protein breakdown for cellular regulation. Here we report the discovery of a new essential component of this degradation machinery. We found the *Saccharomyces cerevisiae* protein Cic1 attached to 26S proteasomes playing a crucial role in substrate specificity for proteasomal destruction. Whereas degradation of short-lived test proteins is not affected, *cic1* mutants stabilize the F-box proteins Cdc4 and Grr1, substrate recognition subunits of the SCF complex. Cic1 interacts *in vitro* and *in vivo* with Cdc4, suggesting a function as a new kind of substrate recruiting factor or adaptor associated with the proteasome.

Keywords: Cdc4/Cic1/proteasome/protein degradation/SCF

Introduction

The ubiquitin–proteasome pathway plays a key role in post-translational regulation in the eukaryotic cell. In degrading non-functional proteins generated under normal or especially under stress conditions, it prevents cells from possible damage. Additionally, it fulfills regulatory functions in adapting cells to developmental, transcriptional and metabolic changes. Moreover, the cell cycle progression of the eukaryotic cell is achieved by degrading cyclins, inhibitors of cyclin-dependent protein kinases, and other regulators in an exactly timed manner (for reviews see Hilt and Wolf, 1996; Hershko and Ciechanover, 1998; Patton *et al.*, 1998b). These proteins are marked for degradation by the attachment of ubiquitin chains. This ubiquitylation process is accomplished by the concerted action of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin–protein ligase (E3).

After ubiquitylation, proteins are degraded by the proteasome (Heinemeyer *et al.*, 1991). This 26S multi-subunit ATP-dependent protease consists of the cylinder-shaped proteolytically active 20S core complex and two

additional regulatory 19S subcomplexes attached to each end of the four-layered 20S cylinder (Baumeister *et al.*, 1998). The crystal structure of the 20S core particle in yeast shows that seven different α -type subunits form the outer layers and enclose the two central rings composed of seven different β -type subunits each. Three of the β -type subunits carry the proteolytically active sites (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997; Groll *et al.*, 1997; Heinemeyer *et al.*, 1997). The precise functions of the α -type subunits are still less clear. They mediate the contact with the 19S subcomplex and are involved in substrate gating together with the 19S regulators (Groll *et al.*, 2000). Of the 18 subunits that build the 19S complex, only Rpn10 has been shown to bind multiubiquitin chains. Since Rpn10 is not essential for yeast growth (van Nocker *et al.*, 1996), other ubiquitin-binding proteins in the 19S cap or alternative mechanisms for efficient transfer of substrates from the ubiquitylation machinery to the proteasome might exist. Supporting the latter possibility, studies in yeast and mammalian cells suggested that the ubiquitylation machinery is not only functionally but also physically connected to the 26S proteasome (Kleijnen *et al.*, 2000; Verma *et al.*, 2000; Xie and Varshavsky, 2000).

In order to unravel the functions of the α -ring in proteasomal degradation, we applied the two-hybrid technique choosing the α -type subunit α 4/Pre6 as a bait to identify interacting proteins. With the two-hybrid approach, we expected to detect components of either putative unknown regulators, assembly factors or proteins engaged in the intracellular localization and distribution of the proteasome. We found a predicted protein of unknown function. We named the encoding gene *CIC1* (core-interacting component). Cic1 is associated with fully assembled 26S proteasomes. It is an essential protein and is localized in the nucleus. The general ubiquitin–proteasome-dependent pathway of protein degradation is not affected in *cic1* mutant cells, but two F-box proteins, Cdc4 and Grr1, are stabilized. We could demonstrate that Cic1 interacts with Cdc4 *in vivo* and *in vitro*, implying a role for Cic1 as an adaptor between the SCF complex and the 26S proteasome.

Results

Identification of a new essential proteasome-interacting protein

We performed a two-hybrid screen with the C-terminus of Pre6 (amino acids 147–253) fused to the Gal4-binding domain as bait. We used Gal4-Pre6 (amino acids 147–253) because fusion of the entire Pre6 to the Gal4-binding domain induced a severe growth phenotype. We found a plasmid containing a DNA fragment in the open reading frame (ORF) of the previously uncharacterized gene

Fig. 1. Cic1 interacts with Pre6. (A) The Cic1 C-terminus is necessary and sufficient for interaction with Pre6 (amino acids 174–253) in the two-hybrid system. Yeast cells expressing the indicated *GAL4* gene fusion were lysed in the presence of X-gal. Dark colony patches result from interaction of the plasmid-encoded proteins. Pre6 (amino acids 147–253) interacts with the C-terminus of Cic1 (amino acids 309–376) and with full-length Cic1 (amino acids 1–376) but not with C-terminally truncated Cic1 (amino acids 1–316). (B) Cic1 interacts with Pre6 *in vitro*. GST–Pre6 was purified from *E. coli* with glutathione–Sepharose (Pharmacia). Immobilized GST–Pre6 and GST were incubated with *in vitro* translated [³⁵S]methionine-labeled Cic1. Cic1 binds to immobilized GST–Pre6 but not to GST. (C) Cic1 is essential for vegetative growth. Sporulation and tetrad dissection of a heterozygous *CIC1/cic1Δ* strain containing pSJ32 (*CEN URA3 CIC1*). All spores can grow on YPD and Sc-uracil plates. The spores containing the kanamycin deletion marker (growth on YPD G418) cannot grow on plates supplemented with 5-FOA, indicating that they cannot lose the *URA3*-marked plasmid containing *CIC1*. (D) Sequence similarities between Cic1, human Pbk1 (PIR accession No. T08693), yeast Ykr060w and a hypothetical protein (PIR accession No. T39179) from *S. pombe*. The sequences were aligned using the ClustAl program (HUSAR Version 3.0, DKFZ Heidelberg, Germany). Gaps (indicated by dots) were used to maximize alignments. Residues identical in at least two of the proteins are shaded in black.

YHR052w. We named this gene *CIC1* (core-interacting component). The identified Gal4 activation domain fusion contained the last 67 amino acids of Cic1. Gal4–Pre6 (amino acids 147–253) also interacted with a full-length Cic1 (amino acids 1–376) fusion whereas a truncated Cic1 fusion (amino acids 1–316), which lacks the last 60 amino acids, did not interact in the two-hybrid system (Figure 1A). This indicates a specific interaction of the Cic1 C-terminus with Pre6.

Next we confirmed the interaction of Pre6 and Cic1 *in vitro*. An immobilized GST–Pre6 (amino acids 1–253) fusion was incubated with *in vitro* translated, radioactively labeled Cic1. Figure 1B shows that Cic1 binds specifically to GST–Pre6 and does not bind to GST alone.

To investigate the *cic1* null phenotype, we generated a heterozygous *CIC1/cic1Δ* diploid yeast strain by deleting one copy of the entire ORF. This strain was transformed with the *CIC1* gene on a *URA3*-based plasmid and

Fig. 2. Cic1 is associated with the 26S proteasome. (A) Cic1 co-precipitates with proteasomal components. Extracts from cells expressing HA3::CIC1 (YS70) were subjected to immunoprecipitation with anti-HA antibodies chemically cross-linked to protein A–Sepharose. HA3-Cic1 was pulled down and the precipitate was analyzed by immunoblotting with anti-Pre6, anti-Sc11, anti-Pre4 and anti-Cim5 antibodies as indicated. The proteasomal subunits are present in the complex with precipitated HA3-Cic1 but not in a strain with untagged Cic1 (YS60). (B) Cic1 co-fractionates with the 19S subunit Cim5. An extract of the wild-type strain W303 was fractionated by gel filtration on Superose 6. The upper curve shows the proteasomal chymotrypsin-like activity in the relevant fractions (12–30). The void volume (v_0) and the marker proteins are indicated by dashed lines. The fractions were analyzed by SDS–PAGE, followed by immunodetection, with anti-Cic1, anti-Cim5, anti-Pre6 and anti-Pre4 antibodies. (C) A strain expressing HA3::CIC1 (YS70) shows the same distribution of HA3-Cic1 as Cic1 in the wild-type strain. (D) Analysis of Superose 6-fractionated proteasomal complexes from YS70 [HA3-Cic1; see (C)] by non-denaturing gel electrophoresis. Following western transfer, immunodetection was performed with anti-Pre6 and anti-Cim5 antibodies, respectively (left panel). Both blots were stripped and reprobed with anti-HA antibodies (right panel) to detect HA3-Cic1 that is only present in the 26S proteasome (RP₂CP).

sporulated. After tetrad dissection, all four spores were viable on YPD and Sc-uracil media. The spores carrying the *cic1Δ* allele were not able to grow on media supplemented with 5-fluoro-orotic acid (5-FOA), which is converted to a toxic product, 5-fluorouracil, by the action of the *URA3* gene product (Figure 1C). Hence, loss of the complementing *URA3/CIC1* plasmid in *cic1Δ* mutants results in non-viable cells. These experiments substantiate that Cic1 function is required for vegetative growth.

Figure 1D shows the result of database searches that revealed homologs of Cic1 in human (overall identity 25% and similarity 35%; in a 100 amino acid stretch the identity reaches 40% and similarity reaches 53%) and *Schizosaccharomyces pombe* (33% identity, 52% similarity) and as well as a homolog in *Saccharomyces cerevisiae* (23% identity, 44% similarity).

Cic1 is a component of the 26S proteasome

Using the genetic two-hybrid approach and *in vitro* binding experiments, we had shown that Cic1 interacts with Pre6. We wished to clarify whether Cic1 is associated with free Pre6, precursor forms of the 20S proteasome, the mature 20S proteasome or the 26S proteasome. To investigate the Cic1–Pre6 interaction in more detail, we performed co-immunoprecipitation experiments. We constructed a strain containing an N-terminally hemagglutinin (HA)-tagged version of Cic1 (strain YS70). A single copy

of the modified *CIC1* gene (HA3::CIC1), expressed from its natural promoter, restored wild-type growth rates (data not shown). Precipitation of HA3-Cic1 with anti-HA antibodies allowed the detection of HA3-Cic1 in the precipitate. This signal was specific, since no immunogenic material was detected in a strain expressing untagged *CIC1*. The 20S proteasome β -type subunit Pre4 (Hilt *et al.*, 1993), the α -type subunits Pre6 (Heinemeyer *et al.*, 1994) and Sc11 (Balzi *et al.*, 1989), as well as a component of the 19S complex, the AAA-ATPase Rpt1/Cim5 (Ghislain *et al.*, 1993), co-precipitated with HA3-Cic1 (Figure 2A). This indicates that Cic1 is present in a complex with the 20S proteasome and at least one subunit of the 19S regulator.

To produce independent evidence of whether Cic1 is part of proteasomal complexes, we performed gel filtration on Superose 6. We analyzed whole-cell extracts of a wild-type strain (W303) and the strain with the HA-tagged version of Cic1 (YS70). Both Cic1 and HA3-Cic1 co-eluted in fractions 14–21 together with the 19S regulator subunit Rpt1/Cim5. Furthermore, these fractions contained the β -type subunit Pre4 and the α -type subunit Pre6 (Figure 2B and C; in Figure 2C, data for Rpt1/Cim5, Pre6 and Pre4 are not shown). Hence, Cic1 is a component of a high molecular weight complex that most probably includes components of the 20S proteasome and its 19S regulatory complex. Cim5 as a component of the 19S particle should be present in fractions containing 26S

Fig. 3. Cic1 localizes to the nucleus and is concentrated in a crescent-shaped part. Fixed and permeabilized YS70 cells were incubated simultaneously with 12CA5 (directed against the HA epitope of Cic1; upper row, left panel) and anti-Pre6 antibodies (lower row, left panel). 12CA5 was visualized with ALEXA₄₈₈-conjugated goat anti-mouse IgG, and anti-Pre6 antibodies with ALEXA₅₄₆-conjugated goat anti-rabbit IgG. DNA was stained with DAPI (upper row, right panel). The middle panel (upper row) shows a merge of antibody staining patterns of 12CA5 and DAPI. The middle panel (lower row) shows a merge of anti-Pre6 and 12CA5 staining. Untagged Cic1 wild-type cells show no staining with 12CA5 (data not shown).

proteasomes with associated proteins as well as pure 26S proteasomes, but should also be found in fractions containing free 19S particles and complexes of 20S proteasomes with only one 19S regulator. The slight shift of the Cim5 elution profile to higher molecular weight fractions compared with that of the 20S core components Pre4 and Pre6 might reflect the fact that a considerable portion of the proteasomal complexes in the cell represents 20S core particles or that cell lysis leads to rapid partial dissociation of the 26S complex. Interestingly, the Cic1 protein content peaked in faster migrating fractions (14–16) than did the Cim5 content (fractions 16–18). This shift of the Cic1 peak away from the Cim5 peak and from the peak of maximum activity can be explained by the binding of Cic1 as a homomeric or heteromeric complex to the 26S proteasome whereby a larger portion of the 26S proteasome remains uncomplexed. In order to identify the proteasome species to which Cic1 binds, we separated the protein complexes of the fast migrating fractions 14–17 of strain YS70 (HA3-Cic1) under non-denaturing conditions. Detection with anti-Pre6 antibodies showed three forms of complexes, the 20S proteasome (CP), the 20S proteasome with one 19S complex (RP₁CP), and the 26S proteasome (RP₂CP) (Figure 2D). Cim5 is present in the 26S form (RP₂CP) and in the RP₁CP complex. In contrast, HA3-Cic1 is only detectable in the 26S form (RP₂CP). These experiments strongly suggest that Cic1 is associated only with fully assembled 26S proteasomes, yielding a proteasomal subpopulation.

To gain more insight into Cic1 function, we determined the localization of a functional HA3-Cic1 fusion (strain YS70) by immunofluorescence microscopy. The signal obtained with anti-HA antibody co-localized with nuclear

Fig. 4. Cic1 is not necessary for general proteasomal degradation. (A) *cic1-2* mutant cells (YS23) grow like wild-type (YS60) at 25°C, but show a strong growth phenotype at the restrictive temperature of 37°C. (B) Cic1 is not required for general proteasomal degradation. Pulse-chase analysis of Leu-β-gal and Ub-Pro-β-gal degradation in YS60 and YS23. Early logarithmically growing cultures were shifted to 37°C for 8 h prior to labeling with [³⁵S]methionine. Multiubiquitylated β-gal species are denoted with open-ended brackets. Asterisks indicate a stable 90 kDa cleavage product characteristic for short-lived β-gal-derivatives. The open square marks stable Pro-β-gal resulting from partial hydrolysis of Ub-Pro-β-gal. Quantification reveals no stabilization of the substrates in *cic1-2* cells.

DNA. There is an enhancement of the signal in the crescent-shaped part of the nucleus where 4',6-diamidino-2-phenylindole (DAPI) stains poorly, i.e. the nucleolus (Figure 3, upper panel). To confirm that this part is indeed the nucleolus, we probed cells expressing a C-terminal Cic1–green fluorescent protein (GFP) fusion (strain YS100) with antibodies against GFP and the nucleolar marker Nop1 (Schimmang *et al.*, 1989) in a double labeling experiment. The signals obtained co-localized in

Fig. 5. Cic1 function is required for turnover of cell cycle regulatory proteins. **(A)** Cdc4 is stabilized in a *cic1-2* mutant. Pulse-chase analysis of degradation of Cdc4 in wild-type (YS60.3) and *cic1-2* mutant cells (YS23.3). Early logarithmically growing wild-type and *cic1-2* mutant cells, expressing HA3-Cdc4 under control of the *GAL1* promoter, were shifted to 37°C for 8 h prior to labeling with [³⁵S]methionine. Quantification reveals an ~2.5-fold stabilization of Cdc4 in the *cic1-2* mutant. **(B)** Grr1 is stabilized in a *cic1-2* mutant. Pulse-chase analysis of degradation of Grr1 in wild-type (YS60.3) and *cic1-2* mutant cells (YS23.3). Early logarithmically growing wild-type and *cic1-2* mutant cells, expressing HA-Grr1^{ΔN} under control of the *ADH1* promoter, were shifted to 37°C for 8 h prior to labeling with [³⁵S]methionine. Quantification shows that the half-life of Grr1 in the *cic1-2* mutant is increased ~2.5-fold. **(C)** Cic1 is not involved in ubiquitylation of Cdc4. Cultures of strain YS23.3 expressing only HA-ubiquitin, Flag-Cdc4 and untagged ubiquitin, or Flag-Cdc4 and HA-ubiquitin, and strain YS60.3 expressing Flag-Cdc4 and HA-ubiquitin were shifted to 37°C for 8 h prior to precipitation with anti-FlagM2 affinity gel. The precipitate was analyzed by immunoblotting with anti-HA antibodies. Both strains show the same ubiquitylation pattern of Cdc4. To confirm that the high molecular weight bands are due to ubiquitylation, we analyzed precipitates of YS60.3 expressing HA-ubiquitin, Flag-Cdc4 and untagged ubiquitin, or Flag-Cdc4 and HA-ubiquitin, with anti-Flag and anti-HA antibodies. With anti-Flag antibodies, the difference in molecular weight between ubiquitin residues and HA-tagged ubiquitin residues bound to Flag-Cdc4 can be detected. With anti-HA antibodies, only Flag-Cdc4 with HA-tagged ubiquitin residues can be detected.

the nucleolar part of the nucleus, indicating that Cic1 is enriched in the nucleolus (data not shown). To see whether there is an overlapping fraction of Cic1 with a bona fide proteasomal subunit, we performed a double staining experiment with anti-HA (detects HA3-Cic1) and anti-Pre6 antibodies in the strain YS70. Regardless of the nucleolar enrichment of Cic1, there is a clear overlap of the Cic1 nucleoplasmic fraction and Pre6 (Figure 3). Hence, the interaction of Cic1 and the proteasome appears to take place in the nucleus.

Cic1 is not required for general proteasomal degradation

To investigate further the function of Cic1, we generated temperature-sensitive *cic1* alleles. Figure 4A shows the growth phenotype of mutant strain YS23 containing the *cic1-2* mutation. The mutation was recessive (data not shown) and *cic1-2* cells were viable at the permissive temperature of 25°C but were not able to grow at the restrictive temperature of 37°C.

To clarify whether the *cic1-2* mutation affects general protein degradation by the proteasome, we tested the degradation kinetics of model substrates. Pulse-chase analyses in a *cic1-2* mutant under restrictive conditions revealed no stabilization of short-lived test proteins such as the N-end-rule substrate Leu-β-galactosidase (Leu-β-gal) that contains the N-terminal leucine as a destabilizing residue (Varshavsky, 1996), and Ub-Pro-β-gal, a substrate of the UFD (ubiquitin fusion degradation) pathway (Johnson *et al.*, 1995) (Figure 4B). We also found no stabilization of the MATα₂₁₋₆₈-β-galactosidase fusion protein (data not shown), a substrate of the DOA (degradation of α2) pathway (Hochstrasser and Varshavsky, 1990). These results show that *cic1-2* proteasomes are fully degradation competent and therefore exclude a role for Cic1 in assembly of the 26S proteasome or as a general regulator of proteasomal activity.

Cic1 is involved in degradation of the substrate recognition subunits Cdc4 and Grr1

The fact that Cic1 is not generally required for proteasomal degradation does not rule out a Cic1 function in degradation of specific proteasomal substrates. Since the need for long incubation times at restrictive temperature made pre-synchronization experiments impossible, we could only test the degradation kinetics of substrates that are degraded cell cycle independently. Recently, it was shown that F-box proteins, e.g. Cdc4, are unstable components of the SCF complex and are degraded rapidly and constitutively via the ubiquitin-proteasome system during the cell cycle (Zhou and Howley, 1998; Galan and Peter, 1999; Mathias *et al.*, 1999). The SCF complex is the E3 enzyme required for G₁-S transition and regulation of sulfur metabolism (Barral *et al.*, 1995; Patton *et al.*, 1998a; Skowrya *et al.*, 1999). The minimal SCF ubiquitin ligase complex is built up of four subunits: Cdc53, Hrt1, Skp1 and the E2 enzyme Cdc34. Further exchangeable subunits, the F-box proteins, are bound to Skp1 via the so-called F-box domain (for a review see Deshaies, 1999). Besides this Skp1-binding site, many of the F-box proteins contain separate protein-protein interaction domains to recruit protein substrates for ubiquitylation (Skowrya *et al.*, 1997; Patton *et al.*, 1998b). The authors suggest that the regulation of F-box protein concentration, hence SCF complex composition, may be critical for a flexible response to cell cycle states and environmental changes.

We investigated the degradation kinetics of the F-box protein Cdc4 in a pulse-chase experiment. Since it was impossible to detect a chromosomal C-terminally HA-tagged version of Cdc4 under its own promoter, we constructed N-terminally HA-tagged Cdc4 under the control of the *GAL1* promoter replacing the endogenous copy of *CDC4* (Longtine *et al.*, 1998).

Fig. 6. Cdc4 interacts with Cic1. (A) Cdc4 interacts with Cic1 *in vitro*. Flag-Cdc4 was precipitated with an anti-FlagM2 affinity gel from a *pre1-1 pre2-2* strain expressing Flag-Cdc4. The precipitate was incubated with *in vitro* translated [³⁵S]methionine-labeled Cic1. The precipitate from cells carrying the expression vector alone was used as a control. Cic1 binds to immobilized Flag-Cdc4. (B) Cdc4 interacts with Cic1 *in vivo*. Extracts from cells expressing Flag-Cdc4 and expression vector, respectively, were subjected to immunoprecipitation with anti-FlagM2 affinity gel. The precipitated proteins were analyzed by immunoblotting with anti-Cic1 and anti-Pre6 antibodies.

We determined the half-life of Cdc4 in the wild-type as ~3 min and in the *cic1-2* mutant as 8 min, indicating an ~2.5-fold stabilization of Cdc4 in *cic1-2* mutant cells (Figure 5A). Furthermore, we examined the stability of a second F-box protein using an HA-tagged version of Grr1 that lacks the N-terminal 280 amino acids. This protein previously has been demonstrated to be fully active since it can complement a *grr1* null allele (Li and Johnston, 1997; Zhou and Howley, 1998). Quantification also revealed an ~2.5 increase of the half-life of Grr1 in a *cic1-2* mutant in comparison with the wild-type (Figure 5B). These data suggest a function for Cic1 in degradation of a distinct subset of regulating proteins.

Ubiquitylation of Cdc4 is independent of Cic1 function

Ubiquitylation of Cdc4 seems to be a prerequisite for degradation of Cdc4 via the proteasome system, probably by an autocatalytic process that requires the core components of the SCF complex, Cdc53 and Skp1 and the E2 enzyme Cdc34 (Zhou and Howley, 1998; Galan and Peter, 1999). To test whether the deficiency in Cdc4 degradation is due to a lack of ubiquitylation in *cic1-2* cells, we investigated the ubiquitylation pattern of Cdc4 in the *cic1-2* mutant in comparison with the wild-type. Flag-Cdc4 and HA-tagged ubiquitin were introduced into the wild-type and the *cic1-2* mutant strain. Flag-Cdc4 was immunoprecipitated, and subsequent immunoblot analysis

of the precipitate with anti-HA antibodies detected the same high molecular weight forms of ubiquitylated Cdc4 in the *cic1-2* cells as in the wild type (Figure 5C).

Cdc4 interacts with Cic1 *in vitro* and *in vivo*

We showed that loss of Cic1 function disturbs neither the general proteasomal function nor the ubiquitylation of the substrate Cdc4. Therefore, Cic1 might act between the ubiquitin system and the proteasomal degradation machinery. A possible scenario could be that Cic1 influences degradation of Cdc4 via a direct interaction. We investigated this possibility in an *in vitro* experiment. Immobilized Flag-Cdc4 was incubated with *in vitro* translated radioactively labeled Cic1. Whereas Cic1 bound to Cdc4, no signal was detected in the vector control; see Figure 6A. We also confirmed this *in vitro* interaction *in vivo*. As shown in Figure 6B, Flag-Cdc4 was immunoprecipitated and we could also detect Cic1 in the precipitate. Additionally, we could co-precipitate the proteasomal component Pre6. These experiments indicate that Cic1 function in degradation of Cdc4 is due to a direct interaction of these proteins in a complex with the proteasome.

Discussion

Here, we describe the identification of a novel and essential proteasome-interacting protein, Cic1. While the general ubiquitin–proteasome pathway is not disturbed in *cic1* cells, our experiments identify Cic1 function to be involved in degradation of two F-box proteins, Cdc4 and Grr1. Moreover, Cic1 interacts directly with the substrate Cdc4.

Cic1 is a novel proteasomal-interacting protein

Using the two-hybrid approach, we found a new proteasomal-interacting protein, that we termed Cic1. By means of several independent biochemical techniques, (i) *in vitro* interaction using purified components, (ii) *in vivo* co-immunoprecipitation and (iii) gel filtration analysis, we thoroughly evaluated that Cic1 is in fact part of proteasomal complexes. Pulse-chase experiments uncovered the half-life of Cic1 to be >1 h, excluding that Cic1 itself is a short-lived protein (data not shown). Cic1 was not identified in the purification of the 19S core complex from budding yeast (Glickman *et al.*, 1998) and in 26S purification experiments (Verma *et al.*, 2000). Like Cic1, a number of additional proteins so far have been reported to be associated with the 26S proteasome (Fujimuro *et al.*, 1998; Schaubert *et al.*, 1998; Kaiser *et al.*, 1999; Papa *et al.*, 1999; Russell *et al.*, 1999a; Verma *et al.*, 2000; Xie and Varshavsky, 2000). This either implies a flexible association of these additional factors with the 26S proteasome, or they represent specific subunits of 26S proteasomal subpopulations. In addition, several viral proteins have been reported to interact with proteasomal ATPases or with subunits of the 20S complex (Huang *et al.*, 1996; Rousset *et al.*, 1996; Berezutskaia and Bagchi, 1997). These viral proteins most probably act in manipulating the proteasomal activity, possibly by taking advantage of existing binding sites for the host's own proteasomal modifiers. It should be emphasized that Cic1 binding to proteasomal complexes via Pre6 is

restricted to the fully assembled 26S (RP₂CP) form, and thus Cic1 cannot be a genuine 19S subunit. Hence, Cic1 binding most probably requires a cross-talk of both Pre6 subunits due to conformational changes of the 20S α -type rings brought about by association of both 19S regulatory particles.

A chromosomally expressed Cic1–GFP fusion and a plasmid-encoded HA3–Cic1 version were found to be located in the nucleus and in the nucleolus. In yeast, proteasomes are located throughout the cell cycle almost exclusively in the nucleus (Enenkel *et al.*, 1998; Russell *et al.*, 1999b). Since we could detect an association of Cic1 with chromatin in chromosomal spread experiments (data not shown), the nucleolar part of Cic1 is probably, directly or by virtue of other proteins, bound to rDNA. According to our immunolocalization studies, we conclude that the proteasomal interaction with Cic1 most probably occurs only in the nucleoplasm and that the nucleolar part could represent sequestered Cic1 as was reported for other proteins (Garcia and Pillus, 1999; Weber *et al.*, 1999).

The role of Cic1 in proteasomal degradation

Using model substrates of the ubiquitin–proteasome pathway, we excluded a general degradation defect of this system in *cic1* mutant cells. This ruled out a Cic1 action in 26S proteasome assembly or as a general proteasomal modifier. However, we found that the degradation of the F-box protein Cdc4 is significantly retarded in *cic1* cells. Subsequent analysis showed that the stabilization of Cdc4 is not due to a defect in ubiquitylation. Thus, *cic1* mutants are competent to form functional SCF^{Cdc4} complexes, excluding a function for Cic1 in SCF complex formation. Consistent with these observations, the SCF^{Cdc4} substrate Sic1 is not stabilized in *cic1* mutants (data not shown). However, the degradation kinetics of the second F-box protein Grr1 are impaired.

Efficient F-box protein degradation may play a key role in regulating SCF complex formation *in vivo* (Deshais, 1999). This would enable cells to respond quickly to different cellular and environmental tasks. Consistent with the observed phenotypes, Cic1 is involved in the regulation of F-box protein abundance. Could Cic1 act as an adaptor recruiting Cdc4 and Grr1 for degradation? Since in *cic1-2* mutants general proteasomal function and Cdc4 ubiquitylation are not affected, Cic1 function must be required for an additional step in between the ubiquitin system and the proteasomal degradation machinery. Experiments providing direct biochemical support for our hypothesis are the observed interaction of Cic1 with Cdc4, implying that Cic1 acts as a factor that binds specific substrates, e.g. Cdc4, and links them to the degradation machinery. Despite the fact that the SCF complex is physically connected to the 26S proteasome (Verma *et al.*, 2000), we could not demonstrate that Cic1 interacts with SCF components other than the substrate recognition subunits, e.g. Cdc4. However, this is in accordance with the fact that the SCF complex most probably binds to the 19S regulatory particle (Verma *et al.*, 2000). Thus, Cic1 might act as a second docking point recruiting the SCF substrate recognition subunits for their subsequent removal. Therefore, the interaction of Cic1 with the holo-SCF complex might be transient and, consequently, not detectable.

The function of an adaptor could reside in ensuring the reliable and fast degradation of certain important regulatory proteins out of the multitude of ubiquitylated substrates, which, especially under stress conditions, flood the proteolytic machinery. Moreover, Rpn10, the as yet only identified 19S subunit that binds polyubiquitin conjugates, plays a non-essential, substrate-specific role in protein turnover and is not necessary for degradation of the bulk of short-lived proteins (van Nocker *et al.*, 1996). It is thus conceivable that the existence of additional factors such as Cic1 guarantee ongoing substrate turnover.

Materials and methods

Cloning and yeast techniques

We constructed a plasmid into which chromosomal *CIC1* with 2 kb of upstream and 1 kb of downstream region was inserted by homologous recombination. A 3.1 kb *EcoRI*–*XbaI* fragment was subcloned in pRS315 and pRS316 to obtain pSJ60 and pSJ32, respectively. HA3::CIC1 was obtained by cloning an HA triple epitope into a *NotI* site introduced directly after the *CIC1* start codon to the resulting plasmid pSJ70. For construction of the two-hybrid fusion plasmid pSJ1, the *HincII*–*BamHI* fragment of *PRE6* was cloned into pAS2 (Bai and Elledge, 1996) digested with *SmaI*–*BamHI*. The ORF of *CIC1* was amplified by PCR and cloned in pGAD-C2 (James *et al.*, 1996), yielding plasmid pSJ75. A fragment containing the C-terminally truncated version of *CIC1* was amplified by PCR, introducing a Stop codon, and cloned into pGAD-C1 to obtain pSJ82.

Standard yeast techniques were used to manipulate strains. The yeast strain Y190 (Bai and Elledge, 1996) and a genomic two-hybrid library (James *et al.*, 1996) were used for two-hybrid studies. The assay was done as described (Bai and Elledge, 1996). Other strains were derivatives of either WCG4 α (MAT α *his3-11,15 ura3 leu2-3,112 can GAL*) or W303 (MAT α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-11 ura3-1*). *CIC1* was deleted with the *KAN^R* marker as described in Wach *et al.* (1994). The primers used were: 5'-I.23-KAN: 5'-AGCAGGCAGATAAACCTACGTTCCAGACTATAAAGTATAAACGTACGCTGCAGGTCGAC-3' and 3'-I.23-KAN: 5'-AAGAAAAAATGAGAGAAAAGATAGATAAGGAGGAAACAAAATCGATGAATTCGAGCTCG-3'. The *cic1 Δ* strain was complemented with pSJ32, resulting in the strains YS32 (WCG4 α background) and YS32.3 (W303 background). YS70 (WCG4 α *cic1 Δ ::KAN^R/pSJ70*) resulted from plasmid shuffling in YS32. To generate *cic1* temperature-sensitive mutant alleles, low-fidelity PCR was used to mutagenize the entire ORF. A gap plasmid was obtained by dropping out a 0.85 kb *BglIII* fragment from pSJ60 and introduced together with the PCR products into strain YS32. The transformed colonies were transferred to 5-FOA media to select for loss of the *URA3*-based plasmid pSJ32, transferred to YPD and then tested for temperature-sensitive growth. Plasmid pSJ23 contains the *cic1-2* allele. The strains YS23 (WCG4 α *cic1 Δ ::KAN^R/pSJ23*) and YS23.3 (W303 *cic1 Δ ::KAN^R/pSJ23*) were obtained by plasmid shuffling in YS32 and YS32.3, respectively. The corresponding wild-type strains YS60 (WCG4 α *cic1 Δ ::KAN^R/pSJ60*) and YS60.3 (W303 *cic1 Δ ::KAN^R/pSJ60*) were created analogously.

The HA-tagged *CDC4* was created by integrating a His3MX6-PGAL1-3HA cassette as described (Longtine *et al.*, 1998). We used the primers F4-CDC4: 5'-ACAGTATTCTTCTTCTTCTCTCATTAACCTTGTTGCGGTTCCGAATTCGAGCTCGTTAAAC-3' and R3-CDC4: 5'-GGAACAGGGATATCACGTAATGGAACCTACAGCTAAGGGAAACGACCCGCACTGAGCAGCGTAATCTG.

Plasmid pYES-Flag-Cdc4 was a gift from P.M.Howley (Zhou and Howley, 1998), and HA-Grr1^{AN} was from M.Johnston (Li and Johnston, 1997).

Anti-Pre6p and anti-Cic1p antibodies, co-immunoprecipitations, gel fractionation, native gels and immunoblots

DNA fragments encoding the entire Pre6 and Cic1 were cloned into expression plasmids pGEX-2T and pGEX-4T3 (Pharmacia), respectively. The GST fusion proteins were purified according to the manufacturer's instructions from *Escherichia coli*, and GST was cleaved off with thrombin. The purified proteins were used for immunization of a rabbit according to standard procedures (Eurogentech).

For co-immunoprecipitation, YS70 or YS60 cells were grown in YPD at 30°C to a density of OD₆₀₀ = 0.5–1. Then 200 OD₆₀₀ units of washed cells were resuspended in TNEG buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 5% glycerine, 1 mM aminoethylbenzene sulfonyl fluoride (AEBSF), complete inhibitor cocktail (Boehringer)] and lysed by vortexing with glass beads on ice. The cell lysate was adjusted to 1% Triton X-100 and incubated for 30 min on ice. After a low speed centrifugation step (10 min, 5000 g, 4°C), the supernatant was pre-cleared with protein A–Sepharose (30 min, 4°C). For immunoprecipitation, anti-HA antibodies covalently bound to protein A–Sepharose were used (Harlow and Lane, 1988). Cell lysates were incubated for 2 h at 4°C. The beads were washed five times with TNEG containing 0.1% Triton X-100 and resuspended in sample buffer. Strains expressing Flag-Cdc4 under control of the *GALI* promoter were grown on SC galactose medium to an OD₆₀₀ = 0.5–1. Then 100 OD₆₀₀ units were lysed by vortexing on ice in 300 µl of TNI buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), complete inhibitor cocktail, 20 mM *N*-ethylmaleimide (NEM), 50 µg/ml E-64, 50 µM *p*-chloromercuriphenylsulfonic acid, 1 mM iodoacetamide]. Cell debris was removed by low speed centrifugation (2000 g, 10 min, 4°C). The supernatant was diluted with 1 ml of TNI buffer (TNI with 0.625% Triton X-100) and centrifuged for 25 min at 13 000 g (4°C). Immunoprecipitation of Flag-Cdc4 was carried out using anti-FlagM2 affinity gel (Sigma) for 90 min at 4°C. The precipitates were washed and resuspended in sample buffer. For the *in vitro* interaction studies, GST–Pre6 was purified from *E.coli* according to the manufacturer's instructions (Pharmacia). GST–Pre6 and precipitated Flag-Cdc4, respectively, were incubated in PBST (phosphate-buffered saline + 0.5% Triton X-100, 90 min, 4°C) with *in vitro* translated [³⁵S]methionine-labeled Cic1. The beads were washed five times with PBST and resuspended in sample buffer. The *in vitro* translation was performed according to the manufacturer's recommendation (Promega). For *in vivo* ubiquitylation, we followed the protocol of Ellison and Hochstrasser (1991). Gel fractionation and assay for chymotrypsin-like activity with fluorogenic peptide substrates in the presence of ATP were performed as described (Ramos *et al.*, 1998). Non-denaturing 4.5% acrylamide gel electrophoresis was performed as described (Hough *et al.*, 1987). The primary antibodies were mouse monoclonal 12CA5 (Babco), mouse monoclonal anti-FlagM2 (Sigma), rabbit anti-Pre6, rabbit anti-Pre4 (M.Fischer and W.Hilt, unpublished), rabbit anti-Cim5 (Ghislain *et al.*, 1993), rabbit anti-Sc11 (U.Gerlinger and W.Hilt, unpublished) and rabbit anti-Cic1. Anti-Pre4 and anti-Pre6 antisera were purified using isolated 20S proteasome transferred to nitrocellulose. Anti-Cic1 antiserum was purified as described in Pringle *et al.* (1991). Pulse–chase analyses were carried out as described (Bachmair *et al.*, 1986).

Cytological techniques

Cells were prepared for immunofluorescence analysis as described (Pringle *et al.*, 1991). For double staining of HA3–Cic1 and Pre6, mouse anti-HA and affinity-purified rabbit anti-Pre6 antibodies were used. For double staining of Cic1–GFP and Nop1, mouse monoclonal anti-Nop1 (gift of C.Enenkel) and affinity-purified rabbit anti-GFP antibodies (gift of M.Knop) were used. A Leica DMX microscope was used and deconvolution was performed as described (McNally *et al.*, 1999).

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