Quality Control of a Cytoplasmic Protein Complex

CHAPERONE MOTORS AND THE UBIQUITIN-PROTEASOME SYSTEM GOVERN THE FATE OF ORPHAN FATTY ACID SYNTHASE SUBUNIT Fas2 OF YEAST

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Mario Scazzari1,2, Ingo Amm1, and Dieter H. Wolf3

From the Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany

For the assembly of protein complexes in the cell, the presence of stoichiometric amounts of the respective protein subunits is of utmost importance. A surplus of any of the subunits may trigger unspecific and harmful protein interactions and has to be avoided. A stoichiometric amount of subunits must finally be reached via transcriptional, translational, and/or post-translational regulation. Synthesis of saturated 16 and 18 carbon fatty acids is carried out by fatty acid synthase: in yeast Saccharomyces cerevisiae, a 2.6-MDa molecular mass assembly containing six protomers each of two different subunits, Fas1 (β) and Fas2 (α). The (α)6(β)6 complex carries six copies of all eight enzymatic activities required for fatty acid synthesis. The FAS1 and FAS2 genes in yeast are unlinked and map on two different chromosomes. Here we study the fate of the α-subunit of the complex, Fas2, when its partner, the β-subunit Fas1, is absent. Individual subunits of fatty acid synthase are proteolytically degraded when the respective partner is missing. Elimination of Fas2 is achieved by the proteasome. Here we show that a ubiquitin transfer machinery is required for Fas2 elimination. The major ubiquitin ligase targeting the superfluous Fas2 subunit to the proteasome is Ubr1. The ubiquitin-conjugating enzymes Ubc2 and Ubc4 assist the degradation process. The AAA-ATPase Cdc48 and the Hsp70 chaperone Ssa1 are crucially involved in the elimination of Fas2.

Enzyme complexes in cells assemble after synthesis of their respective partners. Heterologous enzyme complexes composed of different subunits face the problem that their partners have to be present in stoichiometric amounts to build up the active entity. Any surplus of a subunit that cannot be assembled into the functional complex may possibly be harmful to the cell because it might end up in mistaken interactions with different proteins. In addition, denaturation of the unassembled subunit species might take place, leading to aggregation and precipitation within the cell. In mammals, such conditions lead to severe neurological diseases or diabetes and cancer (1–3). Therefore the amount of any subunit of a heterologous enzyme complex has to be tightly regulated. This can be achieved on the levels of transcription and translation, as well as on the level of proteolysis. Fatty acid synthase is one of the house- hold enzymes of the cell responsible for the de novo synthesis of fatty acids. In yeast it is an enzyme complex of 2.6 MDa composed of six subunits each of two different subunit species, α (Fas2) and β (Fas1). The respective genes FAS1 and FAS2 are unlinked and map on two different chromosomes. Fas1 and Fas2 expression is regulated by several transcriptional activators, as well as by Fas1-mediated autoregulation of Fas2 (4–6). Fine tuning of the subunits is finally exerted by proteolysis (7). Interestingly orphan α subunit Fas2 was shown to be degraded by the proteasome, whereas orphan β-subunit Fas1 ended up in the vacuole (7). Here we followed the fate of orphan Fas2 to unravel the details of its post-translational regulation and elimination mechanism.

EXPERIMENTAL PROCEDURES

Growth Conditions, Yeast Strains, and Plasmids—Genetic and molecular biology techniques were carried out using standard protocols (8–10). To enable growth of fatty acid auxotrophic strains, YPD medium was supplemented with 0.03% myristic acid and 1% Tween 40 (YPD + FA). Liquid synthetic complete minimal medium (CM) was supplemented with 0.03% myristic acid, 1% Tween 40, and 0.05% yeast extract (YCM + FA). When not otherwise indicated, Saccharomyces cerevisiae strains are based on the genetic background W303-1B (MATa, leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15). Generation of gene deletions and epitope tagging were performed via homologous recombination (11–13). Yeast strains used in this study are given in Table 1. The Ubr1-expressing plasmids used in this study were a kind gift from Du et al. (14) and Xie and Varshavsky (15). These plasmids express N-terminally Flag-tagged Ubr1 and an inactive RING1 mutant of Ubr1 (Flag-Ubr1C122D) under control of the ADH1 promoter. For the ubiquitination assays, the plasmid pJD421 expressing

*This work was supported by the Deutsche Forschungsgemeinschaft.
1 These authors contributed equally to this work.
2 Present address: Inst. für Biochemie und Molekularbiologie der Universität Freiburg, Stefan-Meier-Str. 17, 79104 Freiburg, Germany
3 To whom correspondence should be addressed: Inst. für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany. Tel.: 49-711-6856-4371; Fax: 49-711-6856-4392; E-mail: dieter.wolf@ibc.uni-stuttgart.de.
histidine-tagged ubiquitin (His$_6$-Ub) under control of a CLIP1 promoter was used (16), as well as pLA18, a variant of pJD241 possessing the Yeplac195 backbone. It was constructed by inserting the HindIII fragment of pJD241 into the plasmid Yeplac195 (17).

**Antibodies and Western Blotting**—For immunoprecipitation of Fas2 in pulse-chase analysis and detection of Fas1 and Fas2 in Western blot analysis, polyclonal rabbit Fas antibody was used (7). For immunodetection, monoclonal mouse Flag antibody (clone M2; Sigma-Aldrich), monoclonal mouse phosphoglycerate kinase antibody (clone 22C5; Molecular Probes), monoclonal mouse HA antibody (clone 12B12; Covance), rabbit gluco-6-phosphate dehydrogenase antibody (Sigma-Aldrich), and rabbit TAP antibody (Open Biosystems) were purchased. Rabbit Cdc48 antibody was a gift from T. Sommer. Fas2 protein transfer from SDS gel to nitrocellulose was done by wet tank blotting at 200 mAh for 3–4 h on ice with prechilled blotting buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol, 0.02% (w/v) SDS).

**Pulse-Chase and Cycloheximide-Chase Analyses**—Pulse-chase experiments to analyze the degradation of orphan Fas2 were performed as described in (18, 19). Briefly, cells were grown to mid-log phase in YPD + FA media or in selective YCM + FA (for plasmid selection) media and shifted to starvation media (non-selective or selective CM + FA, without sulfate) for 50 min. 50 A$_{600}$ of cells were labeled with 20 μl of [35S]methionine (0.37 MBq/μl; PerkinElmer Life Sciences) for 20 min. Upon addition of fatty acid-supplemented medium containing an excess of nonradioactive methionine, samples were taken at the indicated time points, and cell extracts were prepared. When not otherwise indicated, plotted data represent the mean values of three independent experiments. The error bars represent the respective standard deviation of the mean. Cycloheximide-chase experiments for monitoring the degradation of orphan Fas2 were performed as described by Park et al. (20) with the exception that cells were grown in fatty acid supplemented yeast peptone dextrose media (YPD + FA).

**Ubiquitination Assay**—For detection of ubiquitinated Fas2, 50 A$_{600}$ of cells of each yeast strain transformed with a His$_6$-Ub expressing plasmid were harvested and resuspended in 800 μl of lysis buffer (6 mM guanidine HCl, 100 mM NaHPO$_4$/NaH$_2$PO$_4$, pH 8.0, 10 mM imidazole, 250 mM NaCl, 0.5% (v/v) Nonidet P-40). After lysis using glass beads, the cell lysate was cleared by centrifugation at 15,000 rpm for 15 min. 100 μl of washed Ni-NTA-agarose (Qiagen) were added to each lysate. After 3 h of incubation, the beads were centrifuged at 2000 rpm for 1 min and washed two times with lysis buffer and in addition three times with washing buffer (50 mM NaHPO$_4$/NaH$_2$PO$_4$, pH 8.0, 250 mM NaCl, 20 mM imidazole, 0.5% (v/v) Nonidet P-40). Proteins were eluted by adding 100 μl of urea sample buffer containing 250 mM imidazole and boiling at 80 °C for 10 min. Each supernatant was subjected to SDS-PAGE with subsequent immunoblotting.

**Solubility Assay**—To analyze the solubility of Fas2 substrate in different yeast strains, 20 A$_{600}$ of exponentially grown cells were harvested and resuspended in 1 ml of sorbitol buffer (0.7 M sorbitol, 50 mM Tris-Cl, pH 7.5) containing protease inhibitor mixture (Roche), 1 mM PMSF, and 1.46 μM pepstatin A. Temperature-sensitive strains were shifted to 37 °C for 1 h prior to harvesting. After addition of glass beads, the cells were lysed by vortexing for 15 min at 4 °C. The crude lysate was centrifuged at 500 × g for 5 min. For preparation of the total protein sample (T), 400 μl of the precleared lysate were subjected to TCA precipitation. The pellet was washed once with acetone and solubilized in 60 μl of urea sample buffer (8 M urea, 5% SDS, 200 mM Tris-Cl, pH 6.8, 0.1 M EDTA, pH 8.0, 0.03% bromphenol blue, 1.5% (v/v) β-mercaptoethanol) by boiling at 95 °C for 5 min.
addition 400 μl of the crude lysate were centrifuged for 15 min at 21,500 × g. The supernatant fraction (S) was treated like the total protein fraction. The pellet fraction (P) was washed once in sorbitol buffer prior to solubilization in 60 μl of urea sample buffer at 95 °C for 5 min. All samples were subjected to SDS-PAGE/immunoblotting.

**TAP Pulldown Assay**—Chromosomally C-terminal TAP-tagged Fas2 was purified using Sepharose beads coated with IgGs (IgG SepharoseTM 6 Fast Flow, GE Healthcare). When not otherwise indicated, all steps were carried out at 4 °C. Briefly, 150 A600 of exponentially grown cells (A600 1.0) were harvested by centrifugation, washed once on ice, and incubated for 15 min in 40 ml of 30 mM ice-cold sodium azide on ice. Subsequently, cells were collected by centrifugation, resuspended in 2 ml of cold extraction buffer (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 0.5 mM EDTA, pH 8.0, protease inhibitor mixture (Roche), 1 mM PMSF, 1.46 μM pepstatin A), and lysed by glass bead beating. Cell lysate was centrifuged at 500 × g for 5 min and retrieved supernatant was equally split into two Eppendorf tubes. To the supernatant was added at a concentration of 7 μg/ml of yeast cell lysate. Cell lysate was centrifuged at 500 × g for 5 min and retrieved supernatant was loaded as input control. For tandem affinity purification-immunoprecipitation (TAP-IP), 1 ml of supernatant was added to 1 ml of equilibrated IgG Sepharose beads and incubated for 3 h on a rotator. Subsequently, cells were collected by centrifugation, resuspended in 2 ml of cold extraction buffer, and 0.5% was loaded onto SDS gel. A portion of supernatant proteins were precipitated by TCA and boiled in urea sample buffer, and 0.5% was loaded as input control. For tandem affinity purification-immunoprecipitation (TAP-IP), 1 ml of supernatant was added to 100 μl of equilibrated IgG Sepharose beads and incubated for 3 h on a rotator. Beads were collected and washed three times with 1 ml of cold extraction buffer and two times with 1 ml of cold TEV cleavage buffer (same as extraction buffer but contained in addition 0.01 mM ZnCl2 and 5 mM -mercaptoethanol but only 0.1% Nonidet P-40). Beads were mixed with 100 μl of TEV cleavage buffer plus 3 μl of TEV protease (Roboklon, 8 units/μl) and incubated with gentle agitation for 2 h at 16 °C. Cleaved proteins were collected, precipitated by TCA on ice, washed once with ice-cold acetone, boiled for 5 min in urea sample buffer, and analyzed by Western blot. Elution of IgG-bound Fas2-TAP, shown in Fig. 4B, was done by boiling IgG beads directly in urea sample buffer for 5 min at 95 °C.

**In Vitro Trypsin Sensitivity Assay**—This assay was adapted from Finger et al. (21) and Prasad et al. (22). Briefly, 10 A600 of exponentially grown cells were collected by centrifugation, washed two times with cold water, and lysed with 600 μl of cold cytosolic buffer (20 mM HEPES, pH 7.4, 14% glycerol, 100 mM KOAc, and 2 mM MgOAc and 400 μl of glass beads for 5 min at 4 °C. After centrifugation at 500 × g for 5 min (4 °C), 400 μl of the supernatant was equally split into two Eppendorf tubes. To one tube, trypsin was added at a concentration of 7 μg/ml. The tube without trypsin served as negative control. During incubation at 30 °C, samples were taken (45 μl) at the indicated time points; proteins were immediately TCA-precipitated on ice and analyzed by SDS-PAGE/immunoblotting using respective antibodies.

**Glycerol Density Gradient Fractionation**—50 A600 of cells were grown to log phase, collected by centrifugation, and washed once with 20 ml of cold water. Cells were resuspended in 520 μl of cold 0.1 M KH2PO4/K2HPO4 buffer (pH 6.9), 280 μl of protease inhibitor mixture (Roche), 10 μl of 0.1 M PMSF, and 10 μl of 1.45 mM pepstatin A. After addition of 300 μl of glass beads, cells were lysed by vortexing for 20 min at 4 °C. After precentrifugation at 500 × g for 5 min at 4 °C, lystate was spun down at 16,200 × g for 20 min at 4 °C. 200 μl of the supernatant was layered on top of a 2-ml linear 10–50% glycerol gradient, which was prepared in 20 ml PIPES, pH 6.8. The sample was centrifuged at 55,000 rpm for 4 h at 15 °C in a Beckman Optima TL ultracentrifuge using a TLS55 rotor. Fractions of 170 μl were collected from the top, and proteins were precipitated with TCA on ice and analyzed by SDS-PAGE/immunoblotting. Fatty acid synthase complex, Cdc48, and glucose-6-phosphate dehydrogenase served as molecular weight marker proteins.

**EGFP Fluorescence Microscopy**—Yeast strains expressing endogenous Fas2-EGFP were grown to early-log phase (A600 0.3) in yeast peptone dextrose media. Fatty acid auxotrophic strain Δfas1 was grown in fatty acid supplemented yeast peptone dextrose media. Three A600 of cells were collected by centrifugation, washed once with, and resuspended in 2 ml of PBS buffer (75 mM NaCl, 53 mM NaH2PO4, 13 mM Na2HPO4). For nuclei staining, 2 μl of Hoechst 33342 (10 mg/ml) was added to the cell suspension and incubated with slight agitation in the dark for 25 min at 30 °C. For cell fixation, 1 ml of the cell suspension was transferred to a 1.5-ml Eppendorf tube, and 121 μl of 37% formaldehyde was added (drop by drop) at a final concentration of 4% and incubated in the dark for additional 40 min at 30 °C on a rotator. Subsequently, cells were washed twice with 1 ml and concentrated in 50 μl of IF buffer (1.2 mM sorbitol, 100 mM KH2PO4/K2HPO4, pH 6.5). 2 μl of fixed cells were mounted with Mowiol on a microscope slide, covered by a cover slide, and sealed with nail polish. To visualize Hoechst 33342-stained nuclei and Fas2-EGFP by confocal laser-scanning microscope (LSM710; Carl Zeiss) fluorophores were excited according to their excitation wavelength with an argon laser (488 nm). Analysis of the obtained data and imaging were performed by AxioVision Rel. 4.8.2 software (Carl Zeiss) and Adobe Photoshop CS. Respective yeast strains with untagged Fas2 served as negative control.

**RESULTS**

**Orphan Fas2 Is an Unstable Protein in Vitro**—To be able to follow the fate of an orphan Fas2 protein easily we deleted the FAS1 gene in cells. This left Fas2 as an orphan protein expressed from its wild type gene. The physiological consequence of this deletion, failure to synthesize saturated fatty acids, and thus cessation of growth of cells, was circumvented by the addition of myristic acid and Tween 40 to the growth medium (7). In a first experiment, we tested the proteinase sensitivity of orphan Fas2 in comparison with the assembled (Fas1)6(Fas2)6 complex in cell extracts. As can be seen in Fig. 1A, while the hexameric (Fas1)6(Fas2)6 complex remains stable, orphan Fas2 is rapidly degraded when trypsin is added to cell extracts. This shows an enhanced sensitivity of the orphan protein as compared with its presence in the complex toward proteolysis, suggesting an altered structure of the orphan protein.

**In Vivo Orphan Fas2 Is Degraded by the Proteasome**—Subsequently, we followed the fate of Fas2 in vivo via cycloheximide-
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Figure 1. Orphan Fas2 is proteolytically susceptible in vitro and its degradation depends on the 26 S proteasome in vivo. A, in vitro trypsin sensitivity assay. Native cell extracts from wild type (FAS1 FAS2) and Δfas1 cells expressing endogenous Fas2 were prepared as described under "Experimental Procedures" and treated with (+) or without (−) trypsin. Samples were taken at the indicated time points, and proteins were precipitated, separated, and analyzed by SDS-PAGE/Western blotting using Fas antibody, which detects Fas1 and Fas2 subunits. Endogenously expressed PGK was used as a control. B, in vivo degradation of orphan Fas2 is dependent on the 26 S proteasome. Cycloheximide-chase experiments of endogenously expressed Fas2 were performed as described under "Experimental Procedures" in the respective control strain (CIM3 Δfas1) and in the respective control strain (CIM3 Δfas1). Samples were taken at the indicated time points and subjected to SDS-PAGE and Western blot analysis. Immunoblots were analyzed with Fas and PGK antibody. PGK served as loading control. The data represent the means of two independent experiments. Error bars indicate the standard deviation of the mean.

Orphan Fas2 Organizes into a High Molecular Mass Structure in the Cytoplasm and Requires an ATPase Motor for Elimination—In wild type cells, the 2.6-MDa fatty acid synthase complex consists of six copies each of Fas1 of 229-kDa mass and Fas2 of 207-kDa mass forming a barrel shape complex. The six Fas2 subunits are organized in a wheel-like structure covered by three Fas1 subunits each above and below the wheel in an axial orientation (26). The question arose if the Fas2 subunit would still assemble into the wheel-like hexamer of 1.2 MDA in the absence of its partner Fas1 or if unassembled orphan Fas2 subunits of 207 kDa would remain. For this purpose, glycerol step gradient centrifugation with cell extracts of wild type and Fas1 deleted cells was performed (Fig. 2A). As can be seen, the wild type Fas complex is localized in a fraction on the bottom of the gradient. In contrast, orphan Fas2 sedimented in several fractions above the bottom fraction of 2.6 MDA. The Fas2 protein band with highest density resided below the band of highest density of Cdc48 with its molecular mass of 540 kDa. No major 207-kDa Fas2 fraction was found in the range of the marker protein glucose-6-phosphate dehydrogenase of 240-kDa mass. Thus, orphan Fas2 does not sediment as a single subunit but as a complex in the range between 2.6 MDA and 540 kDa instead, indicating that it may form the 1.2-MDa wheel-like structure or some other high molecular mass complex. For most proteasomal degradation of protein complexes or membrane-associated proteins, the molecular ATPase motor Cdc48 is required, which pulls the entities apart prior to degradation (27, 28). We therefore tested the involvement of Cdc48 in the degradation of orphan Fas2. Indeed, when following the fate of orphan Fas2 in a conditional Cdc48 mutant (alleles cdc48(T413R)) (29), it can be seen that it is not any more degraded to a considerable extent (Fig. 2B). Next we raised the question of whether Cdc48 acts after or before some ubiquitination event of Fas2. Therefore we performed an ubiquitination assay using a strain containing a temperature-sensitive CDC48 allele. We could not detect any difference in ubiquitination of Fas2 after the switch to the restrictive temperature, indicating an involvement of Cdc48 downstream of ubiquitination of Fas2 (Fig. 2C).

Degradation of Orphan Fas2 Is Triggered by the Ubiquitin Ligase Ubr1 and the Conjugating Enzymes Ubc2 and Ubc4—Obviously Fas2 is ubiquitinated prior to proteasomal degradation. Proteasomal degradation of a protein in most cases requires polyubiquitination brought about by an enzyme system consisting of an ubiquitin activating enzyme E1, ubiquitin-conjugating enzymes E2, and ubiquitin ligase Ubr1 as being involved in Fas2 degradation. Deletion of UBR1 causes a considerable stabilization of Fas2 (Fig. 3A). Ubr1 is an ubiquitin ligase that can only ubiquitinate substrates when carrying an intact RING finger domain (15). Indeed, when mutating the essential cysteine residue of the RING finger domain of Ubr1 to serine (allele ubr1C1220S) (15), degradation of Fas2 is dramatically reduced (Fig. 3B). As additional experiment confirming Ubr1-dependent specific degradation of Fas2 in a Fas1 deficient strain, we compared degradation of Fas2 in its wild type environment where both fatty acid synthase subunits are expressed with the degradation of Fas2 in a mutant strain lacking Fas1 (Fig. 3C). For specific detection of Fas2, it was C-terminally TAP-tagged, leaving the fatty acid synthase complex containing Fas2-TAP subunits fully functional (data not shown). Following Fas2 with TAP antibodies shows that this subunit is stable in the wild type situation (i.e. in the presence of Fas1), whereas it is rapidly degraded in a Ubr1-dependent fashion when Fas1 is missing (Fig. 3C). Strong dependence of the stability of Fas2 on the presence of Fas1 is somehow reflected by
expected to occur when both proteins physically interact. This occurred via the respective antibodies (anti-Fas, anti-Cdc48, and anti-glucose-6-phosphate dehydrogenase). The AAA-ATPase Cdc48 is necessary for the degradation of orphan Fas2. Cycloheximide-chase experiments were performed as described under “Experimental Procedures.” Both CDC48 Δfas1 and cdc48ΔT413R Δfas1 strains were shifted to 37 °C before addition of cycloheximide. Samples were taken at the indicated time points and subjected to SDS-PAGE and Western blot analysis. PGK served as loading control. Immunoblots were done with Fas and PGK antibody. PGK served as loading control. The data represent the means of two independent experiments. Error bars indicate the standard deviation of the mean. C, the Cdc48 machinery acts downstream of the ubiquitination process of Fas2. A Ni-NTA-agarose based pulldown assay was performed using a yeast strain possessing a thermosensitive allele and pUBC4. Pulse-chase analysis, seen in Fig. 3H, showed that single deletion of UBC2 had no inhibitory effect on degradation of orphan Fas2. Subsequently, we tested Ubc4 involvement shown to be required for cytoplasmic quality control of a misfolded protein (20). Also single deletion of this enzyme had no effect on degradation of Fas2 (Fig. 3H). However, the absence of both E2s, Ubc2 and Ubc4, resulted in a considerable delay in degradation of orphan Fas2 (Fig. 3H). Obviously these ubiquitin-conjugating enzymes have overlapping activity concerning ubiquitination of orphan Fas2.

**Hsp70 Is Necessary for Orphan Fas2 Elimination**—It had been shown that a heterologously expressed mammalian orphan protein in yeast (39), as well as misfolded proteins of the cytoplasm (20) or some ERAD substrates exposing cytoplasmic domains (19, 35), require Hsp70 chaperones of the Ssa type for degradation. We therefore tested whether degradation of the innate orphan protein Fas2 was also dependent on members of the Hsp70 family. We tested elimination of orphan Fas2 in a strain deleted in the genes encoding three of the four Hsp70-Ssa
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Our studies shed light on the post-translational regulation and the handling of an innate cellular orphan protein, the α-subunit of fatty acid synthase Fas2. Deletion of the FAS1 gene leaves the Fas2 protein as an orphan α-subunit. As found by glycerol step gradient centrifugation, the Fas2 protein is still able to form a higher molecular mass complex (Fig. 2A), possibly the hexameric wheel-like structure (26), which is the basis for the assembly of the entire fatty acid synthase complex. Fas2 is stable when complexed with Fas1 (Fig. 3C). A first indication for an incomplete structural assembly of an intact fatty acid synthase complex in the absence of the subunit Fas1 is the in vitro sensitivity of the Fas2 subunit toward trypsin digestion (Fig. 1A). As previous studies indicated (7), in vivo an orphan Fas2 subunit is degraded by the proteasome (Fig. 1B).

Interestingly, the degradation kinetics of orphan Fas2 exhibits a rather long half-life of ~2 h (Fig. 1B) This is in contrast to the half-life of permanently misfolded proteins, as for instance ΔssCPY* (carboxypeptidase Y with point mutation (G255R) and without ER import signal sequence), and derivatives thereof forced to locate in the cytosol. Their half-life is in the range of 30 min (20, 32). Also the half-lives of many misfolded proteins of the ER are in the range of 30 min or less (35, 46 – 48). This indicates that orphan Fas2 might not be a misfolded protein after synthesis but might fold properly, forming the basic hexameric wheel-like structure, waiting for the β-subunit (Fas1) to dock onto this assembly. It was therefore of interest to find out which machinery might be responsible for targeting orphan Fas2 to the proteasome. This machinery might have been completely different from the machinery required for targeting permanently misfolded proteins to the proteasome. The fatty synthase complex resides in the cytosol (37). We therefore went on to prove or disprove whether the cytosolic ubiquitin ligase Ubr1, which had recently been found to target misfolded cytosolic proteins via ubiquitination to the proteasome, is involved in targeting orphan Fas2 to proteasomal degradation. This turned out to be indeed the case (Fig. 3, A and B). At least over the time period of 4 h, degradation of orphan Fas2 is nearly completely blocked. A slow unfolding process of Fas2 in the assembled structure might occur, which leads to recognition of the then unfolded subunit species for ubiquitination. Alternatively failure to complex with Fas1 leaves an otherwise hidden specific recognition sequence accessible, which leads to recognition by components of the ubiquitin system to trigger degra-

chaperones (SSA2, SSA3, and SSA4) and carrying a temperature-sensitive allele of the fourth Hsp70 gene, SSA1 (allele ssa1-45), carrying a mutation in the peptide-binding domain (40). As control, a strain deleted in SSA2, SSA3, and SSA4 but carrying wild type SSA1 was used. As can be seen in Fig. 4A, in contrast to the SSA1 wild type, degradation of orphan Fas2 was nearly abolished in the ssa1-45 mutant under restrictive conditions of 37 °C, indicating its essential function in the elimination process. The mutated Ssa1-45 protein did not prevent the interaction of Ubr1 with orphan Fas2 under restrictive conditions (Fig. 4B, lane 12). However, at 37 °C, the ssa1-45 mutation caused a considerable increase of Fas2 protein in the pellet (P) fraction (Fig. 4B, lane 11). To more precisely specify the function of Ssa1 in orphan Fas2 degradation, we performed a Fas2 solubility assay (Fig. 4C). As can be seen, at restrictive temperature (37 °C), the strain expressing the temperature-sensitive ssa1-45 allele showed a dramatic shift of Fas2 from the supernatant (S) toward the pellet (P) fraction when compared with nonrestrictive conditions (25 °C). The precipitated Fas2 material is not ubiquitinated, indicating this Ssa1 function to be independent from ubiquitination (Fig. 4D). Clearly, Ssa1 is essential for ubiquitination of Fas2 to occur (Fig. 4D). Hsp70 chaperones work together with Hsp40 class chaperones, which accelerate the ATPase activity of the Hsp70 partner (41, 42). We therefore tested the involvement of several Hsp40 chaperones in the degradation process of orphan Fas2. From several of the tested species (Apj1, Hdj1, Ydj1, and Xdj1), only the lack of Ydj1 activity, as established with the temperature-sensitive ydj1-151 allele (43), seems to have a very minor effect on degradation of orphan Fas2 (Fig. 4E). Neither deletion of SSA1 nor the inhibition of Hsp90 chaperones by geldanamycin (44, 45) showed any effect on the elimination of orphan Fas2 (data not shown).

FIGURE 3. The role of the E3 RING ligase Ubr1, as well as the E2 enzymes Ub2c and Ub4c, in the quality control process of orphan Fas2. A, pulse-chase experiments of endogenously expressed Fas2 were performed in Δfas1 and ubr1Δfas1 deletion strains. Cells were taken at the indicated time points and lysed, and proteins were immunoprecipitated with Fas antibody. After separation by SDS-PAGE, degradation kinetics of orphan Fas2 were detected and quantified using a PhosphorImager system and ImageQuant software, respectively. The data represent the mean values of four independent experiments. Error bars indicate the standard deviation of the mean. B, pulse-chase experiments of endogenously expressed Fas2 were performed in Δfas1 cells, overexpressing either N-terminally Flag-tagged Ubr1 (Flag-Ubr1) or a RING mutant of Ubr1 (Flag-Ubr1C1220S) from a high copy plasmid. Experimental procedures were as described for A. The data represent the mean values of three independent experiments. Error bars indicate the standard deviation of the mean. C, Fas2 is stable when complexed with Fas1. Cycloheximide-chase experiments were performed using yeast strains endogenously expressing TAP-tagged Fas2 in the wild type or in the Δfas1 deletion background, respectively. Fas2-TAP protein was detected at indicated time points after cycloheximide addition using TAP antibody. PGK served as loading control. D, orphan Fas2 is localized to the cytosol. Wild type and Δfas1 cells expressing endogenous EGP-tagged Fas2 or untagged Fas2 (negative control) were prepared for fluorescence microscopy as described under “Experimental Procedures.” The nucleus was stained with Hoechst 33342. Cells were analyzed by laser-scanning microscopy. E, ubiquitination of Fas2 in absence of Fas1 is dependent on Ubr1. A Ni-NTA–agarose-based pulldown assay was performed using yeast strains expressing histidine-tagged ubiquitin. Eluate and input samples were subjected to SDS-PAGE and immunoblotting using Fas antibody. F, Fas2 in its wild type environment is not ubiquitinated. The ubiquitination assay was performed as described above, using a wild type strain expressing both Fas1 and Fas2 subunits and the FAS1 deletion strain. G, Ubr1 and the RING mutant (Ubr1C1220S) are physically associated with orphan Fas2 in vivo. Chromosomally expressed Fas2-TAP was purified by one-step TAP purification (as described under “Experimental Procedures”) from the Δfas1Δfas1FAS2-TAP mutant strain harboring a high copy plasmid, encoding either N-terminally Flag-tagged Ubr1 (Flag-Ubr1) or a RING mutant of Ubr1 (Flag-Ubr1C1220S). Cells transformed with empty vector and strains in which Fas2 was not tagged with TAP served as negative controls. 0.5% of supernatant prior to incubation with IgG beads was used as input control. Fas2-TAP was pulled down by incubation of the supernatant with IgG beads. Elution of IgG-bound Fas2-TAP was done by TEV protease-mediated cleavage (TEV-C). Eluate and input were subjected to SDS-PAGE followed by immunoblotting. Fas2-TAP and Flag-Ubr1 were detected by Fas and Flag antibody, respectively. PGK detected by PGK antibody served as a negative interaction control. H, pulse-chase experiments of endogenously expressed Fas2 were performed in Δfas1, ubc2Δfas1, ubc4Δfas1, and Δubc2Δubc4Δfas1 cells. Cells were further processed as described in A. The data represent the mean values of three independent experiments. Error bars indicate the standard deviation of the mean. Note: gray vertical line indicates pulse-chase data combined from two different SDS gels.
FIGURE 4. Degradation and solubility of orphan Fas2 is dependent on the Hsp70 chaperone Ssa1 but shows little change in the absence of the Hsp40 chaperone Ydj1. A, cycloheximide-chase experiments of chromosomally expressed Fas2 were performed as described for temperature-sensitive strains under “Experimental Procedures” with the SSA mutants: SSA1 Δfas1 Δssao/Δssao Δssao and ssa1-45 Δfas1 Δssao Δssao, both deleted in FAS1. The Δfas1 strain harboring all four SSA wild-type genes served as an additional control. Samples were taken at the indicated time points and subjected to Western blot analysis. Immunoblots were analyzed with Fas and PGK antibody, whereby PGK served as loading control. The data represent the means of four independent experiments. Error bars indicate the standard deviation of the mean.

B, the in vivo interaction of orphan Fas2 and Ubr1 does not require a direct Ssa chaperone function. Endogenously expressed Fas2-TAP was purified by one-step TAP purification as described under “Experimental Procedures” from a ssa1-45 Δfas1 Δssao Δssao Δssao Δubr1 Δfas1 FAS2-TAP mutant strain containing either a high copy plasmid, expressing C-terminally HA-tagged Ubr1 (Ubr1-HA), or empty vector. Cells were grown at 25 °C and shifted to 37 °C 1 h before harvesting. Cells transformed with empty vector served as negative control. 0.5% of the lysate and the pellet fraction were loaded as input and pellet fractions, respectively. Fas2-TAP was pulled down by incubation of the supernatant with IgG beads (TAP-IP). Elution of IgG-bound Fas2-TAP was achieved by boiling the beads directly in urea sample buffer. All fractions were subjected to SDS-PAGE followed by immunoblotting. Fas2-TAP and Ubr1-HA were detected by Fas and HA antibody, respectively. PGK detected by PGK antibody served as negative interaction control.

C, for the solubility assay of orphan Fas2, 20 μl of cells of the yeast strains containing either a wild-type SSA1 allele or a temperature-sensitive ssa1-45 allele, both deleted in SSA2, SSA3, and SSA4, were harvested prior to or after a temperature shift to 37 °C for 1 h. The cells were lysed, and the lysates were fractionated into total (T), supernatant (S), and pellet (P) fraction followed by TCA precipitation, solubilization, and SDS-PAGE/immunoblotting. The amount of Fas2 in the different fractions was visualized by using Fas antibody. PGK served as soluble reference protein.

D, a Ni-NTA-based pulldown assay for detecting Fas2 ubiquitination was performed using the Δssao Δssao Δssao Δssao deletion strain harboring either the wild type SSA1 gene or the temperature-sensitive ssa1-45 allele. Samples for input and pulldown were taken after 1-h temperature shift from 25 to 37 °C. E, pulse-chase experiments of endogenously expressed Fas2 were performed in YDJ1 Δfas1 and ydj1-151 Δfas1 cells, according to the protocol for temperature-sensitive strains (see “Experimental Procedures”). Samples were taken at the indicated time points and lysed, and proteins were immunoprecipitated with Fas antibody. After separation by SDS-PAGE, degradation kinetics of orphan Fas2 were detected and quantified using a PhosphorImager system and ImageQuant software, respectively. The data represent the mean values of three independent experiments. Error bars indicate the standard deviation of the mean.
The reason why degradation resumes to some extent after 4 h has to be elucidated in future studies. Recently Shemorry et al. (49) showed that the N-terminally acetylated Cog1 subunit of the multimeric COG complex of S. cerevisiae involved in Golgi transport is a target of the Not4 E3 ligase with subsequent degradation by the proteasome. It could be shown that moderately overexpressed Cog1 could be stabilized by co-expression of interacting subunits of the COG complex. Fas2, its amino acid sequence starting with Met-Lys, cannot become N-terminally acetylated (50, 51) and thus cannot become a target of Not4. Proteins containing unacetylated N-terminal methionine can become substrates of Ubr1 if this methionine is followed by a hydrophobic amino acid (52). This is not the case for Fas2. Here the N-terminal methionine is followed by the basic amino acid lysine. Also, methionine cannot be cleaved off the N-terminal Met-Lys sequence (53–55). Thus, orphan Fas2 cannot become a classical N-end rule substrate. Ubr1 must have other means to recognize orphan Fas2. Ubr1 is known to work together with the ubiquitin-conjugating enzyme Ubc2. However, single deletion of UBC2 did not alter the degradation rate of Fas2. Only when combined with an additional deletion of UBC4 was degradation of Fas2 significantly disturbed (Fig. 3H). Clearly, the ligase Ubr1 and the substrate Fas2 interact (Fig. 3G). The requirement of the ATPase motor Cdc48 for elimination of orphan Fas2 underlines the presence of an assembled Fas2 complex prior to degradation: Cdc48 has been found in most instances tested so far to be responsible for tearing ubiquitinated complexes apart prior to proteasomal degradation (28, 29). As found for regulated proteasomal degradation of fructose-1,6-bisphosphatase (29) or proteasomal degradation of cytoplasmically localized misfolded ΔssCPY* species (20), elimination of orphan Fas2 also requires the Hsp70 chaperone Ssa1 (Fig. 4A). Interestingly, a conditional mutant of Ssa1 (Ssa1-45), carrying a mutation in the peptide-binding domain does not disturb the binding between Ubr1 and Fas2 under restrictive conditions (Fig. 4B). This indicates that Ssa1 may not be a linker in the binding process between the enzyme and the substrate unless this function is exerted by an as yet unknown co-chaperone. A major function of Ssa1 seems to reside in keeping orphan Fas2 in a soluble form for subsequent proteasomal degradation (Fig. 4C) as shown for a permanently misfolded ΔssCPY* species (20). Obviously Ssa1 acts prior to ubiquitination of Fas2 because the precipitated material is not ubiquitinated (Fig. 4D). The necessity of Hsp70 chaperones for degradation again shows the importance of substrate solubility for proteasomal degradation to occur. None of the tested Hsp40 co-chaperones, including Ydj1, seems to have a prominent role in the degradation process of orphan Fas2. Either none of them is involved, or they exhibit overlapping functions.

In summary, our findings give insight into the protein quality control of an orphan protein that is subjected to Ubr1 dependent proteasomal degradation. In the proposed model, upon deletion of FAS1 only Fas2 is synthesized and then organizes into a homomeric complex. This complex is kept soluble by the Hsp70 chaperone Ssa1. Subsequently orphan Fas2 is ubiquitinated by Ubr1 with the help of the E2 enzymes Ubc2 and Ubc4, disassembled by the Cdc48 machinery and subjected to proteasomal degradation (Fig. 5). It will be of future interest to elucidate the reason why orphan Fas2 is targeted to the Ubr1-linked ubiquitin proteasome pathway for elimination, whereas orphan Fas1 is directed to the vacuole for degradation (7).

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