Ribosome Association and Stability of the Nascent Polypeptide-Associated Complex Is Dependent Upon Its Own Ubiquitination

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ABSTRACT

In this work we addressed the role of ubiquitination in the function of the *n*ascent polypeptide-*a*ssociated *c*omplex (NAC), named EGD in the yeast *Saccharomyces cerevisiae*. To this end, we first identified the lysines residues required for ubiquitination of EGD/NAC. While simultaneous mutation of many lysines in the α-subunit of NAC (Egd2p) was required to abolish its ubiquitination, for the β-subunit of NAC (Egd1p), mutation of K29 and K30 was sufficient. We determined that the ubiquitination of the two EGD subunits was coordinated, occurring during growth first on Egd1p and then on Egd2p. Egd2p was ubiquitinated earlier during growth if Egd1p could not be ubiquitinated. The use of mutants revealed the importance of EGD ubiquitination for its ribosome association and stability. Finally, our study demonstrated an interaction of EGD/NAC with the proteasome and revealed the importance of the Not4p E3 ligase, responsible for EGD/NAC ubiquitination, in this association.

W HEN emerging from the ribosome, newly synthesized polypeptide chains are immediately bound by molecular chaperones that assist in subsequent folding or by specifically targeting factors that help nascent chains to reach their proper cellular localization (BUKAU *et al.* 2000; HARTL and HAYER-HARTL 2002). In yeast and metazoans, the Hsp70-type chaperone, Ssz, and its DnaJ co-chaperone, Zuotin, form a *r*ibosome-*a*ssociated *c*omplex (RAC) (GAUTSCHI *et al.* 2001; HUNDLEY *et al.* 2005; OTTO *et al.* 2005). In yeast, RAC interacts with another ribosome-bound Hsp70 homolog, Ssb (GAUTSCHI *et al.* 2001; HUNDLEY *et al.* 2002), where only Ssb is in direct association with nascent chains. In addition to the Hsp70-RAC machinery, there is a protein complex, the *n*ascent polypeptide-*a*ssociated *c*omplex (NAC), which associates with ribosomes and nascent chains in an apparent 1:1 stoichiometry (WIEDMANN *et al.* 1994; FUNFSCHILLING and ROSPERT 1999; ROSPERT *et al.* 2002).

NAC is a highly conserved heterodimeric complex composed of two subunits (α and β) that are both in direct contact with nascent polypeptide chains (WIEDMANN *et al.* 1994); however, βNAC alone is responsible for binding to the ribosome (BEATRIX *et al.* 2000). The yeast genome encodes three known NAC homologs: a single α-subunit (encoded by *EGD2*) and two β-subunits (encoded by *EGD1* and *BTT1*), where Btt1p is expressed at 100 times lower levels than Egd1p (GEORGE *et al.* 1998;

REIMANN *et al.* 1999). All NAC homologs contain a NAC domain, responsible for dimerization. Both β-subunits can form heterodimeric complexes with αNAC. Egd1p can also form homodimers (PANASENKO *et al.* 2006), and probably so can Egd2p since archaeal NAC is an αNAC homodimer (SPRETER *et al.* 2005). Although NAC is highly conserved and present in archaea, yeast, and mammalian cells, our knowledge of its function *in vivo* is still far from complete.

It has been shown that NAC associates with the ribosome through binding of the ribosomal protein Rpl25p, near the site where newly synthesized polypeptide chains emerge (WEGRZYN *et al.* 2006). These data, together with the observation that NAC crosslinks to short nascent polypeptides (WIEDMANN *et al.* 1994), have led to the speculation that NAC might play a role in the folding of newly synthesized proteins, protecting them from interaction with inappropriate cytosolic factors. It was proposed that cycles of binding and releasing NAC would expose the polypeptide to the cytosol in quantal units, rather than amino acid by amino acid. NAC would thus contribute to fidelity in cotranslational processes such as targeting and folding (WANG *et al.* 1995). There has also been evidence that NAC directly interacts with the signal recognition particle and is involved in correct translocation of proteins to the endoplasmic reticulum by regulating the accessibility of the translocation pore and by preventing the mistargeting of nonsecretory proteins (LAURING *et al.* 1995; MOLLER *et al.* 1998). In addition, a regulatory role for NAC in the import of proteins into mitochondria was proposed (GEORGE *et al.* 1998;

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TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
MY1	*MATa ura3-52 trp1-1 leu2::PET56 gcn4Δ*	Collart and Struhl (1994)
MY1719	Isogenic to MY1 except *MATα not5::LEU2*	Lenssen *et al.* (2005)
MY3595	Isogenic to MY1 except *MATα not4::KanMX4*	Lenssen *et al.* (2007)
MY3609	Isogenic to MY1 except *egd2::KanMX4*	Panasenko *et al.* (2006)
MY3610	Isogenic to MY1 except *egd1::KanMX4*	Panasenko *et al.* (2006)
MY3622	Isogenic to MY1 except *not4::LEU2 egd1::KanMX4*	This work
MY3624	Isogenic to MY1 except *not4::LEU2 egd1::EGD1-HA₃-KanMX4*	Panasenko *et al.* (2006)
MY3894	Isogenic to MY1 except *egd1::EGD1-HA₃-KanMX4 ubc4::TRP1*	This work
MY3895	Isogenic to MY1 except *egd1::EGD1-HA₃-KanMX4 ubc5::TRP1*	This work
MY3897	Isogenic to MY1 except *not4::LEU2 egd1::EGD1-HA₃-KanMX4 ubc5::TRP1*	This work
MY4272	Isogenic to MY1 except *ubc5::TRP1*	This work
MY4519	Isogenic to MY1 except *not4::KanMX4:NOT4-URA3 egd1::EGD1-HA₃-KanMX4*	Panasenko *et al.* (2006)
MY4644	Isogenic to MY1 except *egd1::NatMX4 egd2::KanMX4*	This work
MY4863	Isogenic to MY1 except *ubc4::NatMX4*	This work
MY5125	Isogenic to MY1 except *not4::LEU2 egd1::EGD1-HA₃-KanMX4 ubc4::TRP1*	This work
MY5561	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 rpn1::RPN1-TapTag-URA3*	Gavin *et al.* (2006)
MY5693	*MATa rpn1::RPN1-TapTag-URA3 not4::KanMX4*	3595 × 5561
MY5699	*MATa rpn1::RPN1-TapTag-URA3*	1719 × 5561

Funfschilling and Rospert 1999); however, direct evidence to support this hypothesis is still lacking. Finally, NAC has also been associated with transcription regulation (Zheng *et al.* 1987, 1990; Quelo *et al.* 2002, 2005; Akhouayri *et al.* 2005) and with human cell differentiation (Lopez *et al.* 2005), mostly in situations of unequal expression of either NAC subunit, suggesting individual functions of the α- and β-subunits.

The biological importance of NAC is highlighted by the embryonic lethality of NAC mutants in mice (Deng and Behringer 1995), nematodes (Bloss *et al.* 2003), and fruit flies (Markesich *et al.* 2000). In contrast, deletion of EGD/NAC in yeast (referred to as EGD from here on) is not lethal and leads to only insignificant growth defects at high temperature (Reimann *et al.* 1999). αNAC contains a *ub*iquitin-*a*ssociated (UBA) domain, which is found in several proteins involved in the ubiquitin–proteasome pathway for protein degradation. The UBA domain is structurally distinct from the NAC domain (Spreter *et al.* 2005) and it is not necessary for heterodimer formation, but it is required for stability of EGD (Panasenko *et al.* 2006). Recently, we found that the Not4p E3 ligase, a component of the nine-subunit evolutionarily conserved Ccr4-Not complex, was responsible for regulated ubiquitination of EGD in yeast and influenced its cellular localization (Panasenko *et al.* 2006). However, the exact role of EGD ubiquitination *in vivo* remains unknown.

In this work, we undertook the identification of the ubiquitinated residues in EGD to investigate the role of ubiquitination for this chaperone. We determined that the ribosome association and stability of Egd1p required its own ubiquitination, particularly in the absence of Egd2p. In addition, we found that Egd2p binds

to the proteasome, a 2.5-MDa protease present in all eukaryotes, which degrades proteins conjugated to ubiquitin. The proteasome can be subdivided into two major subcomplexes: (1) the 20S *c*ore *p*article (CP), a hollow cylinder that consists of a stack of four rings, two outer rings with seven different α-type subunits, and two inner rings with seven different β-type subunits each and (2) two additional 19S *r*egulatory *p*articles (RP; also called PA700 in mammals) attached to the ends of the 20S cylinder to build up the 26S proteasome holoenzyme (Peters *et al.* 1994). In particular, we found that Egd2p associates with the CP, containing the proteolytically active sites of the proteaseome, in a Not4p E3 ligase-dependent manner. This result suggests a role for EGD ubiquitination in its association with the proteasome. Our finding of the importance of EGD Not4p-dependent ubiquitination in its association with the ribosome at the site of nascent chain emergence, on the one hand, and with the proteasome, on the other hand, is provocative. Indeed, ubiquitination is likely to play a role in the targeting of inappropriately folded nascent polypeptides to the proteasome, indicating that the Not4p E3 ligase and EGD are possible candidates for playing a role in this process.

MATERIALS AND METHODS

Media and strains: All media were standard. The strains used in this work derive from MY1 or from the Euroscarf strain SC0000 (Table 1). Single-step deletions and/or tagging of genes were performed by PCR according to Longtine *et al.* (1998).

DNA constructs: Plasmids expressing HA-tagged Egd1p (p*ADH1-EGD1-HA*) and Egd2p (p*ADH1-EGD2-HA*) under the control of the *ADH1* promoter were made by cloning

EGD1 or *EGD2* sequences with an HA tag, amplified by PCR, into a pRS414-derived plasmid containing the *ADH1* promoter (pMAC392; PANASENKO *et al.* 2006) between the *Bam*HI and *Xho*I sites, leading to pMAC594 and pMAC596. Mutagenesis of Egd1p and Egd2p was performed on the basis of pMAC594 and pMAC596 with the standard Promega protocol, leading to plasmids pMAC595 (Egd1$_{K10,13R}$p-HA), pMAC768 (Egd1$_{K29,30R}$p-HA), pMAC604 (Egd1$_{K10,13,29,30R}$p-HA), pMAC629 (Egd1$_{K10,13,29,30,43,50,66R}$p-HA), pMAC624 (Egd1$_{K43,50,66R}$p-HA), pMAC625 (Egd1$_{K138,139R}$p-HA), pMAC636 (Egd1$_{R24A,R25A,K26R}$p-HA), pMAC599 (Egd2$_{K18,19,26R}$p-HA), pMAC626 (Egd2$_{K18,19,26,42,52,70R}$p-HA), pMAC628 (Egd2$_{K42,52,70R}$p-HA), and pMAC600 (Egd2$_{K159R}$p-HA). The marker gene in pMAC768 was changed from *URA3* to *TRP1* by homologous recombination in yeast, leading to pMAC770.

Plasmids expressing Myc$_6$-tagged Ubc4p and Ubc5p were made by cloning *UBC4* or *UBC5* sequences, amplified by PCR, into pGREG516 (JANSEN *et al.* 2005) between the *Sal*I sites, by homologous recombination in yeast. The *GAL1* promoter in these plasmids was changed to the *UBC4* or *UBC5* promoters amplified by PCR and cloned between the *Asc*I and *Not*I sites by homologous recombination in yeast, leading to pMAC640 (p*UBC4-Myc$_6$-UBC4*) and pMAC648 (p*UBC5-Myc$_6$-UBC5*), respectively. The sequences of all plasmids were verified.

Co-immunoprecipitation: A total of 100 OD units of cells grown to an OD$_{600}$ of 0.8 were broken with 1 ml of glass beads in 0.8 ml of an immunoprecipitation (IP) buffer (40 mM HEPES–KOH, pH 7.5, 150 mM K-acetate, 40 mM KCl, 1 mM EDTA, 20% glycerol, 1 mM PMSF) during 15 min at 4°. The supernatant was clarified by centrifugation for 20 min at 14,000 × *g* at 4° and the total protein concentration in the supernatant was measured by the Bradford assay. To analyze the protein levels in the lysates, we loaded on SDS–PAGE gels 12 μg of total protein extract (TE). For immunoprecipitation, 0.4 ml of the lysates containing 2.5 mg of total protein was mixed with 100 μl of 20% Protein A-sepharose and 1 μl of anti-HA antibodies (IP anti-HA) for 10 hr at 4°. For control (IP control), we used the same mixture but without antibodies. After incubation, beads were washed three times with 1 ml of IP buffer and boiled with 50 μl of two times concentrated SDS–sample buffer, 15 μl of which was analyzed by SDS–PAGE. The efficiency of immunoprecipitation was analyzed by Western blot with antibodies against HA. Co-immunoprecipitated proteins were analyzed by Western blot with antibodies against Rpl25p and Egd2p.

Stability assay and *in vivo* ubiquitination assay: These assays were performed as described earlier (PANASENKO *et al.* 2006). Equal loading of the gels in these experiments was verified by ponceau S staining of all membranes prior to Western blotting.

Modeling: To model NAC–ribosome interaction, we use the described structure of the *t*rigger *f*actor (TF) from *Deinococcus radiodurans* interacting with the ribosome, particularly with Rpl23p (SCHLUNZEN *et al.* 2005). To build a homology model of the interaction of Egd1p with Rpl25p, the ribosome-bound state of the TF loop (TF-BD, PDB:2d3o) was used as a template in Swiss-PDB-Viewer (GUEX and PEITSCH 1997). This modeling is supported mainly by a multiple alignment of numerous TF and Egd1p homologous sequences that shows a strong conservation of several residues in the loop region. The three-dimensional structure of the ribosomal proteins interacting with TF (Rpl23p, Rpl24p, and Rpl29p) was superimposed to the low-resolution, three-dimensional structure of their eukaryotic homologs (Rpl25p, Rpl26Ap, and Rpl34p, respectively) in an unbound state (PDB:1s1i) (SPAHN *et al.* 2004).

The model of Egd1p–Egd2p interaction was performed with modeler8 v2 (SALI and BLUNDELL 1993), using as a template the structure proposed for archaeal NAC (aeNAC,

PDB:1tr8) as done previously (SPRETER *et al.* 2005). Reconstruction of the loops and energy minimization were done with Swiss-PDB-Viewer. ANOLEA profiles were computed for each chain as a validation indicator for the model (MELO *et al.* 1997). The secondary structures of Egd1p and TF homologs were predicted using PSIPRED v2.6 (JONES 1999; MCGUFFIN *et al.* 2000).

A protein–protein rigid body docking experiment between Rpl25p-Rpl26Ap ribosomal proteins and ubiquitin was done with Hex v4.5 (RITCHIE and KEMP 2000). The search was performed using full rotation of molecules and was based on both shape and electrostatics.

Proteasome purification: Proteasomes were purified from yeast strains expressing the RP subunit Rpn1p with a tobacco etch virus (TEV)-ProA tag (Tap-tag) at the C terminus as described in LEGGETT *et al.* (2005) with some modifications as outlined below, but all of the buffers were the same. Briefly, cells from 10 liters of culture, grown to stationary phase (OD$_{600}$ of 12), were harvested, washed with buffer 1, resuspended in a twofold volume of the same buffer containing 10% glycerol, and disrupted with glass beads. Lysates were clarified by centrifugation at 43,000 × *g* for 45 min and frozen at −80°. A total of 100 ml of lysates, containing 2 g of total protein, were incubated with immunoglobulin–sepharose (IgG–sepharose) for 1.5 hr at 4° and the resin was washed with 50 bed volumes (BV) of buffer 2. We divided the resin into two parts: one was used for the purification of the holoenzyme and the other for the purification of RP and CP. The holoenzyme was eluted by equilibrating the IgG resin with buffer 5 and then incubating with 1.5 BV of buffer 5, containing 100 units/ml of His$_6$-TEV protease (Invitrogen) at 30° for 1.5 hr. TEV protease was subsequently removed from the eluate by incubation with Ni-NTA resin (Qiagen) at 4° for 15 min. To purify the CP, we incubated the second part of the IgG resin with 5 BV of buffer 3 for 1 hr at 4° and collected the flow through. To purify the RP, resin was washed with 50 BV of buffer 3, equilibrated with buffer 5, and incubated with 1.5 BV of buffer 5, containing 100 units/ml of His$_6$-TEV protease at 30° for 1.5 hr. TEV protease was removed with Ni-NTA resin.

Antibodies: Antibodies against Rpl25p were kindly provided by Elke Deuerling. Production of antibodies against Egd1p and Egd2p was described previously (PANASENKO *et al.* 2006). Antibodies against Rpt1p, Rpt2p, and α1, -2, -3, -5, -6, and -7 were purchased from Biomol.

RESULTS

Ubc4p and Ubc5p play different roles in Egd1p ubiquitination: Ubiquitination of the EGD complex *in vivo* depends upon Not4p (PANASENKO *et al.* 2006), and Not4p can interact in the two-hybrid assay with two yeast E2-conjugating enzymes, Ubc4p and Ubc5p (ALBERT *et al.* 2002; PANASENKO *et al.* 2006). *In vitro* the EGD complex can be ubiquitinated by Ubc4p and Not4p (PANASENKO *et al.* 2006), so we investigated the role of Ubc4p and Ubc5p in ubiquitination of the EGD complex *in vivo* using wild-type or *not4Δ* strains expressing HA-tagged Egd1p and deleted (or did not delete) *UBC4* or *UBC5*. As previously published (PANASENKO *et al.* 2006), ubiquitinated forms of Egd1-HA were detectable in wild-type cells and increased as glucose was depleted from the growth medium (Figure 1A). Deletion of *NOT4* led to reduced Egd1p ubiquitination, as expected, whereas deletion of *UBC4* did not affect
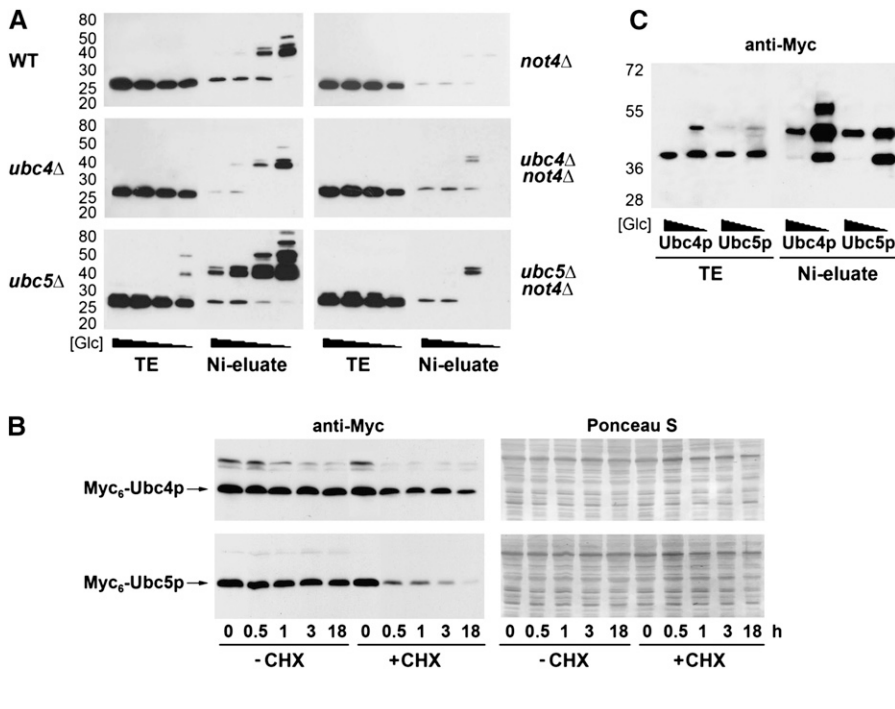
FIGURE 1.—Different roles of Ubc4p and Ubc5p in Egd1p ubiquitination. (A) Total protein extracts were prepared from wild-type (WT), *ubc4Δ*, *ubc5Δ*, *not4Δ*, *ubc4Δ not4Δ*, and *ubc5Δ not4Δ* cells collected from exponential phase to glucose depletion and expressing Egd1p-HA₃ and His₆-ubiquitin. TEs and proteins eluted from a nickel column (Ni-eluate) were analyzed by Western blot with antibodies against the epitope tag. The molecular weights of protein markers are indicated on the left. (B) *ubc4Δ* or *ubc5Δ* strains expressing Myc₆-Ubc4p or Myc₆-Ubc5p were harvested after the indicated times in medium with cycloheximide (CHX, 50 μg/ml). The levels of the tagged proteins (indicated on the left) were revealed by rapid alkaline lysis and Western blot using antibodies against the tag (left), and equal protein loading was verified by ponceau S staining (right). (C) Total protein extracts prepared from *ubc4Δ* or *ubc5Δ* cells expressing Myc₆-Ubc4p or Myc₆-Ubc5p and His₆-ubiquitin were collected from exponential phase to glucose depletion and analyzed as described in A.

Egd1p ubiquitination, which remained mostly Not4p dependent. Surprisingly, Egd1p ubiquitination strongly increased in *ubc5Δ* cells, although it remained Not4 dependent. Unfortunately, we could not measure Egd1p ubiquitination in the double *ubc4Δ ubc5Δ* mutant because the double deletion was not viable in our genetic background.

Previous work has suggested that Ubc4p and Ubc5p are functionally redundant E2 enzymes (CHUANG and MADURA 2005), yet our results now show that Ubc4p and Ubc5p are not functionally identical with regard to Egd1p ubiquitination. In addition, it was reported that Ubc4p is expressed at much higher levels than Ubc5p and is the E2 enzyme primarily detected in actively growing cells (CHUANG and MADURA 2005). However, we observed that deletion of *UBC5* had a more dramatic effect on Egd1p ubiquitination than did the deletion of *UBC4*. Both proteins were expressed at similar levels at the early stage of growth at high glucose concentration in the medium and 18 hr later when glucose had been depleted (Figure 1B). Interestingly, Ubp5p was unstable, with a half-life of <30 min, whereas Ubc4p was much more stable (Figure 1B). Thus, the differences in the cellular levels of Ubc4p and Ubc5p described in previous studies might have stemmed from the difference in their stabilities rather than from differences in their expression. A slower migrating form of Ubc4p, and to a lesser extent of Ubc5p, was visible in our experiments (Figure 1B, left) and compatible with ubiquitination of these E2 enzymes. Indeed, using the same assay described above for Egd1p and Egd2p, we observed that both Ubc4p and Ubc5p were ubiquitinated

(Figure 1C). Ubc4p was more extensively ubiquitinated, yet more stable, than Ubc5p, suggesting that the observed ubiquitination was not associated with destabilization for these E2 enzymes.

Taken together, these results show that the Ubc4p and Ubc5p E2 enzymes are expressed at similar levels but have different stabilities, are both ubiquitinated, and play different roles in EGD complex ubiquitination.

Ubiquitination of the two EGD subunits is tightly coordinated: To investigate EGD ubiquitination further, we tried to identify on which lysine residues the ubiquitination occurs. There are 18 lysine residues in Egd1p and 16 in Egd2p, all of which could potentially be ubiquitinated (Figure 2A). We mutated to arginine the most conserved lysines in Egd1p and Egd2p and performed a ubiquitination assay.

In the case of Egd1p (Figure 2B, top), some mutations, such as K43,50,66R (lane 6, right) or K138,139R (lane 7, right) did not affect its ubiquitination (compare to lane 1, right), whereas mutation of all seven lysine residues, K10,13,29,30,43,50,66R (lane 5, right), led to a striking reduction of Egd1p ubiquitination at both the exponential and post-diauxic phases of growth. K10,13,29,30R and K29,30R had the same reducing effect (lanes 3 and 4, left and right), whereas mutations K10,13R had only a minor impact on Egd1p ubiquitination (lane 2, right). All derivatives were equally expressed (left). These results suggest that ubiquitination of Egd1p might occur on lysines 29 and/or 30.

In the case of Egd2p, only upon mutation of a large number of lysine residues, such as in the mutant K18,19,26,42,52,70R, was there any measurable reduction
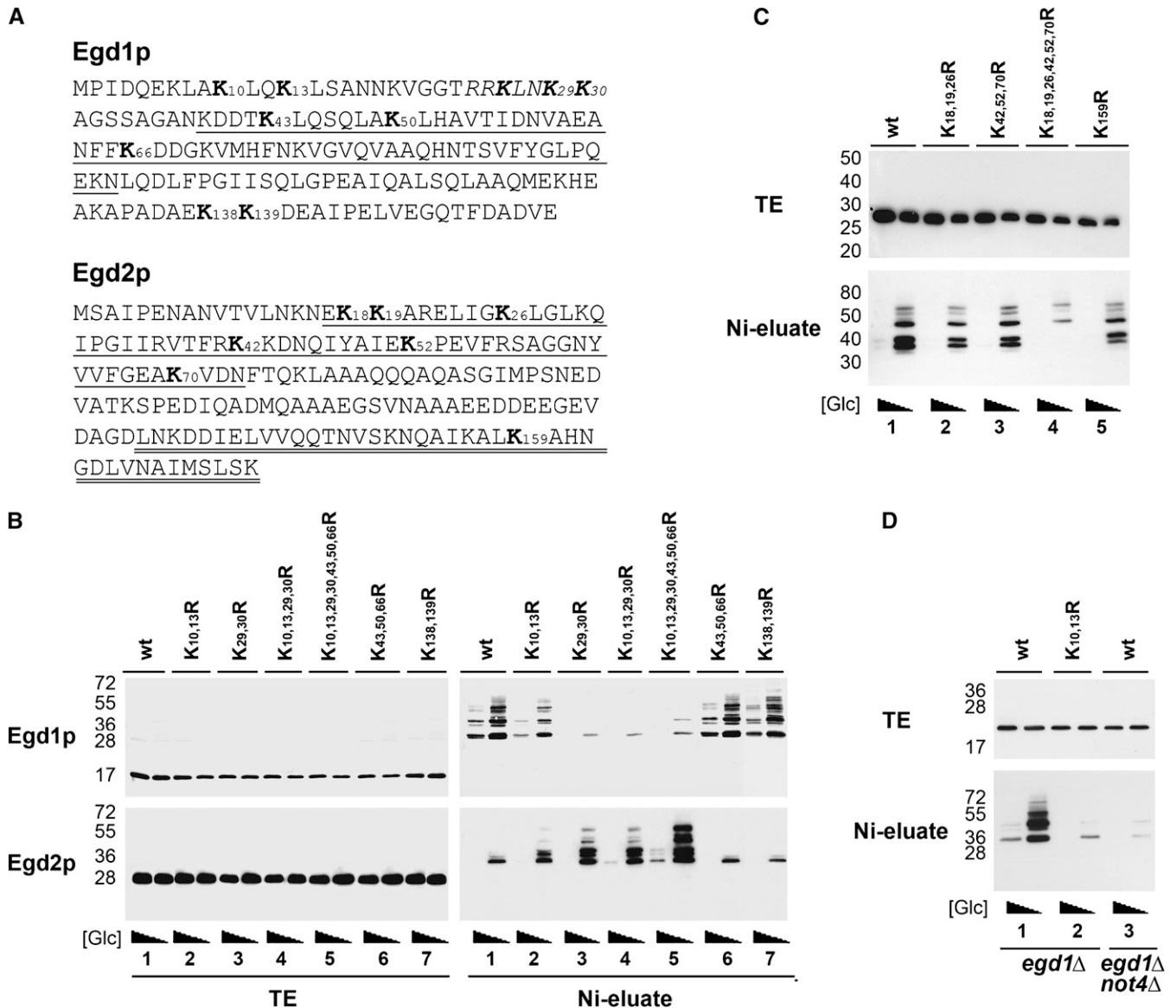
A

Egd1p

MPIDQEKLA**K**₁₀LQ**K**₁₃LSANNKVGGT*RR**K**LN**K**₂₉**K**₃₀
AGSSAGANKDDT**K**₄₃LQSQLA**K**₅₀LHAVTIDNVAEA
NFF**K**₆₆DDGKVMHFNKVGVQVAAQHNTSVFYGLPQ
EKNLQDLFPGIISQLGPEAIQALSQLAAQMEKHE
AKAPADAE**K**₁₃₈**K**₁₃₉DEAIPELVEGQTFDADVE

Egd2p

MSAIPENANVTVLNKNE**K**₁₈**K**₁₉ARELIG**K**₂₆LGLKQ
IPGIIRVTFR**K**₄₂KDNQIYAIE**K**₅₂PEVFRSAGGNY
VVFGEA**K**₇₀VDNFTQKLAAAQQQAQASGIMPSNED
VATKSPEDIQADMQAAAEGSVNAAAEEDDEEGEV
DAGDLNKDDIELVVQQTNVSKNQAIKAL**K**₁₅₉AHN
GDLVNAIMSLSK

FIGURE 2.—Ubiquitination of Egd1p and Egd2p is linked. (A) Primary structure of Egd1p and Egd2p. The most conserved lysine residues are marked in boldface. NAC and UBA domains are underlined with one or two lines, respectively. The mapped ribosome-binding site in the structure of Egd1p is indicated by italics. (B) Total protein extracts were prepared from *egd1Δ* cells collected from exponential phase to glucose depletion and expressing from episomes His₆-ubiquitin and HA-tagged Egd1p, either wild type or carrying the indicated mutations, and analyzed as in Figure 1A. Membranes were analyzed with antibodies against HA (top) or, after stripping, with antibodies against Egd2p (bottom). Molecular weights of protein markers are indicated on the left. (C) Total protein extracts were prepared from *egd2Δ* cells expressing from episomes His₆-ubiquitin and HA-tagged Egd2p, either wild type or carrying the indicated mutations, and analyzed as described in Figure 1A. The signal in C for Egd2p is stronger than in B because different antibodies were used (HA *vs.* Egd2p). (D) Total protein extracts were prepared from *egd1Δ* (lanes 1 and 2) or *egd1Δ not4Δ* (lanes 3) cells expressing His₆-ubiquitin and HA-tagged Egd1p, either wild type (lanes 1 and 3) or K29,30R (lane 2). Cells were grown and collected as described in Figure 1A. Molecular weights of protein markers are indicated on the left.

in Egd2p ubiquitination (Figure 2C, lane 4). These results suggest that Egd2p might be carrying ubiquitin entities on several different lysine residues, rather than a polyubiquitin chain on one given lysine, but it is also possible that ubiquitination can occur on different lysines when the appropriate one is mutated.

During exponential growth, Egd1p, but not Egd2p, was ubiquitinated (compare lane 1 in Figure 2, B and C), whereas both Egd1p and Egd2p were ubiquitinated

after the diauxic shift, most likely in response to depletion of several nutrients, including glucose (data not shown). According to these observations, one can imagine that, in the EGD complex, Egd1p is ubiquitinated first and Egd2p ubiquitination occurs later. Thus, it was interesting to investigate whether there was any correlation between ubiquitination of the two subunits. We followed Egd2p ubiquitination in *egd1Δ* cells expressing wild-type or non-ubiquitinated forms of Egd1p
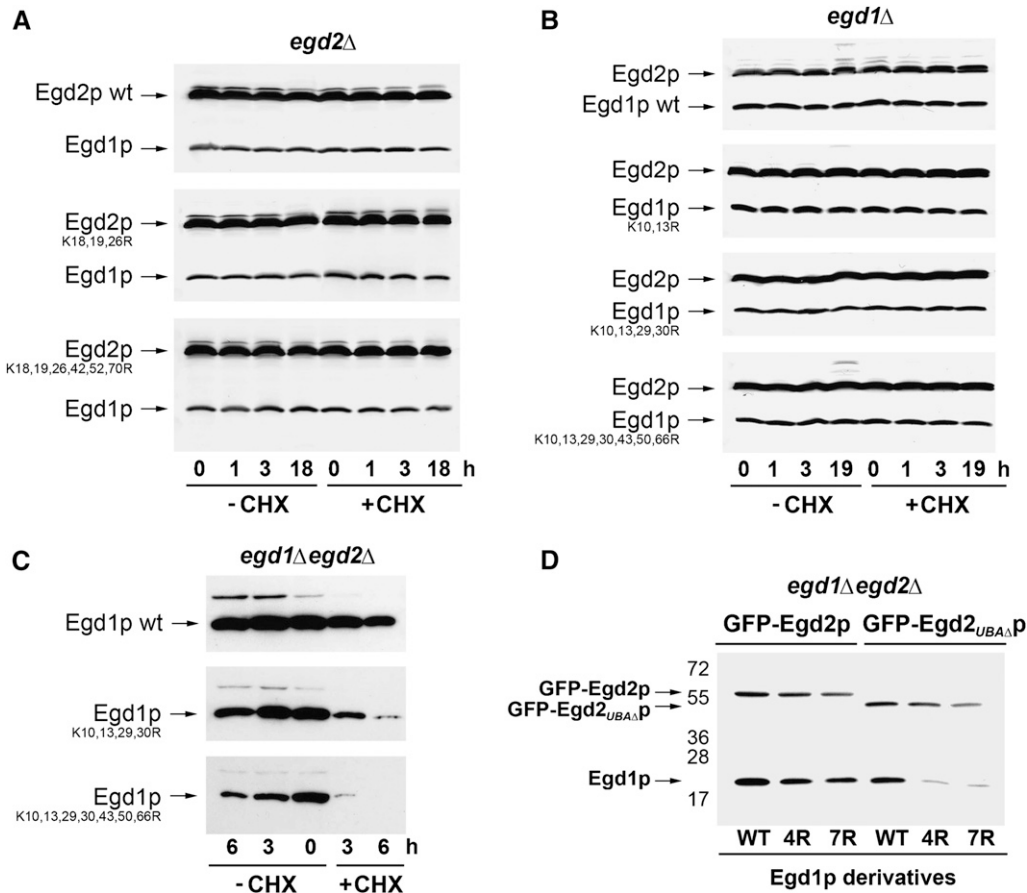
FIGURE 3.—Stability of Egd1p is dependent upon its own ubiquitination and Egd2p. (A) *egd2Δ* cells expressing HA-tagged Egd2p, either wild type or carrying the indicated mutations, were treated and analyzed as in Figure 1B, with antibodies against HA or against Egd1p to reveal endogenous Egd1p. (B) *egd1Δ* cells expressing HA-tagged Egd1p, either wild type or carrying the indicated mutations, were treated and analyzed as in Figure 1B, with antibodies against HA or against Egd2p to reveal endogenous Egd2p. (C) *egd1Δ egd2Δ* cells expressing HA-tagged Egd1p, either wild type or carrying the indicated mutations, were analyzed as in Figure 1B. (D) *egd1Δ egd2Δ* cells expressing GFP-Egd2p or GFP-Egd2$_{ΔUBA}$p and HA-tagged Egd1p, either wild type (WT), K10,13,29,30R (4R), or K10,13,29,30,43,50,66R (7R) were grown to an OD_{600} of 0.8 and collected after 6 hr when the drop of Egd1p in cells totally lacking Egd2p was maximal (C). Total extracts were analyzed by Western blot for the presence of the Egd1p and Egd2p derivatives with antibodies against the HA or GFP tags.

(Figure 2B, bottom). While Egd2p was moderately ubiquitinated in cells expressing wild-type Egd1p—and this only after the diauxic shift (lane 1, left and right)—its ubiquitination was strikingly increased in cells growing exponentially and after the diauxic shift, when they expressed a mutant form of Egd1p that was not ubiquitinated (K29,30R, K10,13,29,30R or K10,13,29,30,43,50,66R) (lanes 3–5). Thus, Egd1p is the major subunit of the EGD complex that is ubiquitinated, but if it cannot be ubiquitinated, Egd2p becomes more extensively ubiquitinated.

As previously reported (PANASENKO *et al.* 2006), ubiquitination of endogenous Egd1p is decreased in *not4Δ* cells (see Figure 1A). Deletion of Not4p similarly abolished ubiquitination of Egd1p expressed from a plasmid, and this to the same extent as mutation K29,30R in Egd1p (Figure 2D: compare lanes 2 and 3 to lane 1).

Stability of Egd1p requires its own ubiquitination and Egd2p: Our results indicate that ubiquitination of the two subunits of the EGD complex is connected, and this raises the question of the role of this ubiquitination. One possibility is that it mediates destabilization of the EGD complex. However, the stable levels of both

subunits were constant during 18 hr of cell growth, with or without cycloheximide, whether the derivatives were ubiquitinated or not (Figure 3, A and B). These observations suggest that the EGD subunits are generally very stable and that their ubiquitination does not mediate their degradation, although one cannot exclude the possibility that only a small proportion of the EGD subunits is ubiquitinated and turned over. Furthermore, the stability of the mutants also suggests that they are probably not misfolded.

Previously, we determined that the presence of Egd2p was required for Egd1p stability (PANASENKO *et al.* 2006), and we thus considered that ubiquitination of Egd1p might be required to degrade Egd1p in the absence of Egd2p. To test this, we compared the stability of wild-type or mutant Egd1p in cells lacking Egd2p. As previously observed, wild-type Egd1p was slightly unstable in the absence of Egd2p with a half life of ~3 hr (Figure 3C, top). Surprisingly, non-ubiquitinated Egd1p derivatives were much less stable than wild-type Egd1p in cells lacking Egd2p (Figure 3C: compare the two bottom panels to the top panel). Furthermore, we noted that, in *egd2Δ* cells, the levels of non-ubiquitinated

Egd1p derivatives decreased during the growth of cells, even in the absence of cycloheximide, at the time of growth when ubiquitination of wild-type Egd1p usually increases. Thus, our results indicate that, in contrast to our initial hypothesis, in egd2Δ cells Egd1p ubiquitination correlates with its stability.

As mentioned above, Egd2p has a UBA domain that is important for Egd2p's own stable expression (Panasenko et al. 2006). To analyze if this UBA domain may influence the stability of Egd1p, we used plasmids expressing N-terminally GFP-tagged Egd2p with and without a UBA domain. Indeed, we observed that the presence of a GFP entity at the N-terminal end of Egd2p was able to stabilize UBA-less Egd2p (Figure 3D) such that, by using fusion proteins, we could compare expressed Egd2p and UBA-less Egd2p to define the role of the UBA domain. We measured wild-type and mutant Egd1p levels in cells expressing GFP-Egd2p or GFP-Egd2$_{\Delta UBA}$p. Wild-type Egd1p was expressed at slightly lower levels in cells expressing UBA-less Egd2p compared to full-length Egd2p. The level of Egd1p mutants was not significantly reduced compared to wild-type Egd1p in cells expressing full-length Egd2p. In contrast, in cells expressing UBA-less Egd2p, the level of Egd1$_{K10,13,29,30R}$p and Egd1$_{K10,13,29,30,43,50,66R}$p was dramatically reduced compared to wild-type Egd1p or compared to the level of these mutants in cells expressing full-length Egd2p [Figure 3D: compare 4R and 7R lanes to wild-type (WT) lanes]. Interestingly, UBA-less Egd2p was also less ubiquitinated than full-length Egd2p (supplemental Figure 1).

Thus, the UBA domain of Egd2p is required not only for stability of Egd2p itself as previously observed, but also for its own ubiquitination and for the stability of non-ubiquitinated Egd1p.

Ribosome association of the EGD complex is dependent upon its ubiquitination, Not4p, and Egd2p: We showed above that Egd1p ubiquitination requires its lysines K29 and K30 and is necessary for Egd1p stability in the absence of Egd2p, but the question of the relevance of this ubiquitination for the cellular function of the EGD complex remains. One clear function attributed to the EGD complex is its association with the ribosome, so we investigated how ubiquitination of the EGD complex might affect its ribosome binding. Indeed, K29 and K30, required for Egd1p ubiquitination as shown above, are part of the ribosome-binding site of Egd1p (RRK(x)nK$_{29}$K$_{30}$), and the mutation K29,30A has been reported to reduce the ribosomal association of Egd1p (Wegrzyn et al. 2006). To thus analyze the role of ubiquitination for ribosome association of Egd1p, we immunoprecipitated wild-type and mutant Egd1p. Egd1p was expressed at roughly the same level in all strains during early exponential growth and was immunoprecipitated with similar efficiency (Figure 4A, left). The ribosomal protein Rpl25p, with which the EGD complex interacts (Wegrzyn et al. 2006),

was expressed at similar levels in all strains and was co-immunoprecipitated with wild-type or mutant Egd1p in cells expressing Egd2p (Figure 4A, middle), although maybe with slightly less efficiency in the case of the non-ubiquitinated derivatives of Egd1p (Figure 4A, middle). In egd2Δ cells, Rpl25p was co-immunoprecipitated with wild-type Egd1p, albeit less efficiently than in the presence of Egd2p (Figure 4A: compare each lane 1 of the middle). In contrast, only traces of Rpl25p were co-immunoprecipitated with Egd1$_{K10,13,29,30R}$p and Egd1$_{K10,13,29,30,43,50,66R}$p. The interaction of Egd2p was similar with wild-type or mutant forms of Egd1p (Figure 4A, right), indicating that ubiquitination of Egd1p is not essential for heterodimer formation and, also again, that the Egd1p mutants are appropriately folded.

Our result indicates that ribosome association of Egd1p is dependent upon its own ubiquitination and Egd2p. As shown above, Egd1p ubiquitination depends upon Not4p (see Figure 1A and Figure 2D), so we analyzed how deletion of Not4p might influence the ribosome binding of Egd1p. Egd1p was expressed at the same level in wild-type and not4Δ strains and was immunoprecipitated with similar efficiency (Figure 4B, left). Rpl25p was also expressed at similar levels in both strains and was co-immunoprecipitated with Egd1p from wild-type cell extracts, but not from cell extracts lacking Not4p (Figure 4B, right). Thus, deletion of Not4p, which reduces ubiquitination of both Egd1p and Egd2p (Panasenko et al. 2006), disrupts Egd1p ribosome association even in the presence of Egd2p.

Loss of Egd1p ubiquitination results in reduced association of Egd1p with its ribosomal partner Rpl25p in the absence but not in the presence of Egd2p. Deletion of NOT4 reduces Egd1p ribosomal association even in the presence of Egd2p, but we know that in this case ubiquitination of both Egd1p and Egd2p is reduced. These results suggest that increased ubiquitination of Egd2p might compensate for reduced Egd1p ubiquitination and support association of the EGD complex with ribosome in the first situation. To test this, we studied Rpl25p binding of a non-ubiquitinated mutant of Egd1p (K29,30R) in cells expressing a mutant of Egd2p (K18, 19,26,42,52,70) that is much less ubiquitinated than wild-type Egd2p after glucose depletion (see Figure 2C). First, we found that, indeed, this mutant Egd2p was much less ubiquitinated than wild-type Egd2p in cells expressing a non-ubiquitinated mutant of Egd1p (Figure 4C). Second, immunoprecipitation of Egd1$_{K29,30R}$p from cells expressing wild-type or mutant Egd2p was equally efficient (Figure 4D, top), but co-immunoprecipitation of Rpl25p was significantly decreased from cells expressing the K18,19,26,42,52,70R mutant of Egd2p compared to wild-type Egd2p (Figure 4D, bottom). These results suggest that increased ubiquitination of Egd2p indeed can compensate for reduced Egd1p ubiquitination to support EGD ribosome association.
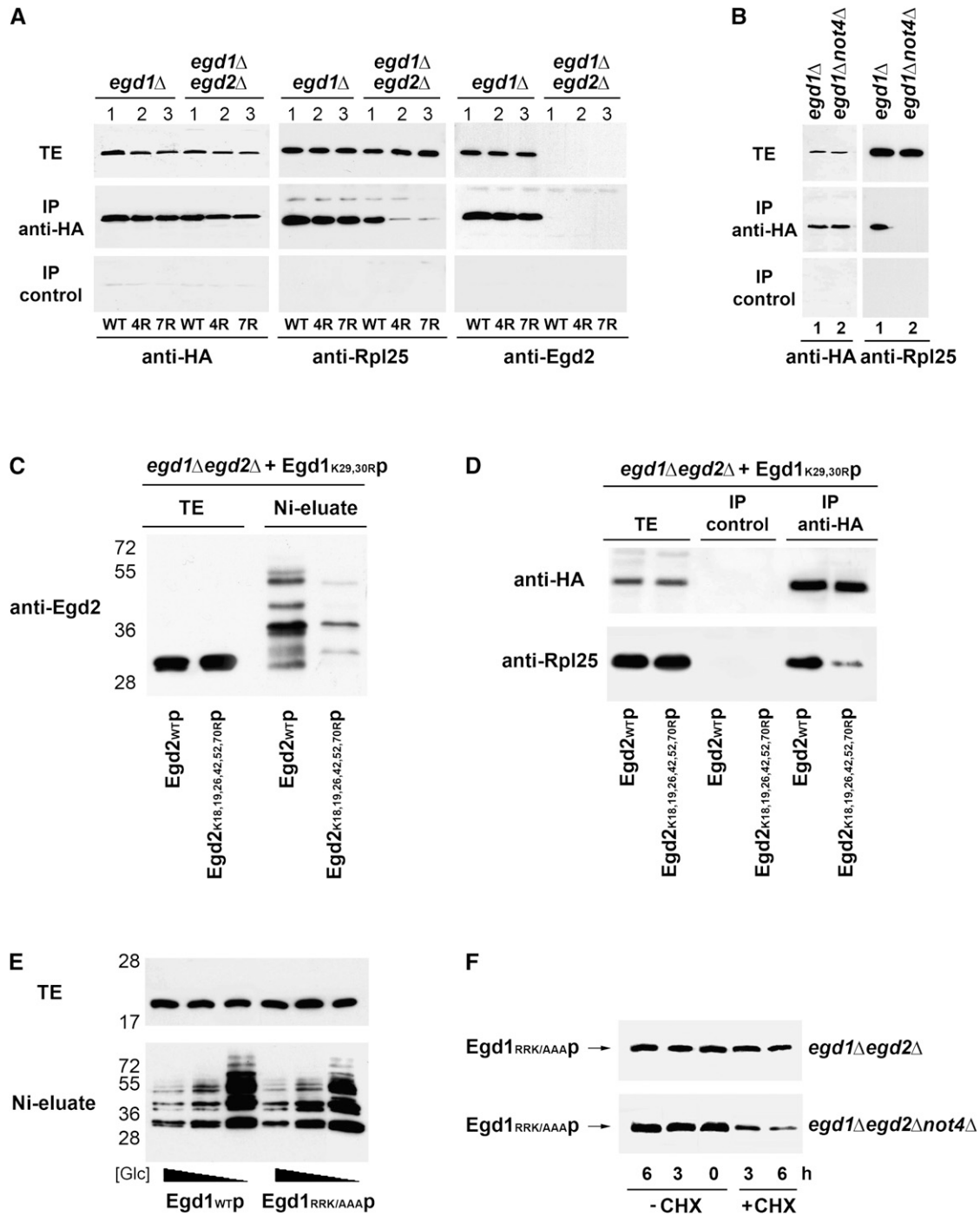
FIGURE 4.—Ribosome association of the EGD complex is dependent upon its own ubiquitination and Egd2p. (A) Egd1p derivatives were immunoprecipitated from total protein extracts of *egd1Δ* or *egd1Δ egd2Δ* cells expressing tagged Egd1p, either wild type (WT, lanes 1), K10,13,29,30R (4R, lane 2, or K10,13,29,30,43,50,66R (7R, lane 3), with antibodies against the HA tag. TE and immunoprecipitates were analyzed by Western blot for the presence of tagged Egd1p (left), Rpl25p (middle), and Egd2p (right) with the antibodies indicated below the panels. (B) Egd1p-HA was immunoprecipitated from *egd1Δ* (lane 1) or *egd1Δ not4Δ* (lane 2) cells. TE and immunoprecipitates were analyzed for the presence of Egd1p-HA (left) or Rpl25p (right). (C) Total protein extracts were prepared from *egd1Δ egd2Δ* cells expressing His$_6$-ubiquitin, Egd1$_{K29,30R}$p-HA, and wild-type or mutant Egd2p-HA as indicated. TE and proteins eluted from a nickel column were analyzed by Western blot with antibodies against Egd2p. The molecular weights of protein markers are indicated on the left. (D) The EGD complex was immunoprecipitated from total protein extracts collected from *egd1Δ egd2Δ* cells expressing Egd1$_{K29,30R}$p-HA and wild-type or mutant Egd2p-HA. TE and immunoprecipitates were analyzed with antibodies against HA or Rpl25p as indicated on the left. (E) Total protein extracts were prepared from *egd1Δ* cells expressing His$_6$-ubiquitin and HA-tagged Egd1p, either wild type (Egd1$_{WT}$p) or mutated in its ribosome-binding domain (Egd1$_{RRK/AAA}$p). The ubiquitination assay was performed as in Figure 1A. (F) *egd1Δ egd2Δ* or *egd1Δ egd2Δ not4Δ* cells expressing HA-tagged Egd1$_{RRK/AAA}$p were analyzed as in Figure 1B for the presence of the mutant Egd1p.
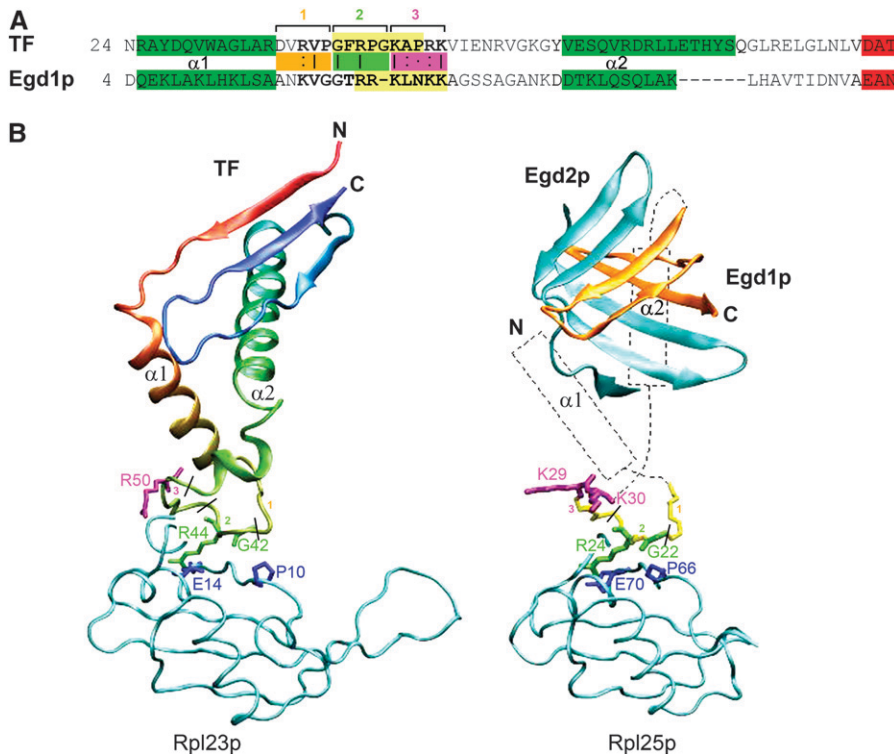
FIGURE 5.—Comparison of the interaction of bacterial TF and yeast EGD with the ribosome. (A) Sequence alignments of *D. radiodurans* TF and *S. cerevisiae* Egd1p was derived from a multiple alignment that confirms the high conservation rate of several residues in this region over homologous sequences of TF and βNAC/Egd1p. The three parts of the ribosome-binding loop are indicated by numbers and orange, green, and purple, respectively. Identical, equivalent, and similar residues are shown with "|" (vertical line), ":" (colon), and "." (period), respectively. Pale yellow indicates the conserved motifs known from the literature (KRAMER *et al.* 2002; WEGRZYN *et al.* 2006). The modeled regions are indicated in boldface type. Predicted α-helices and β-strands are in green and red, respectively. (B) TF is displayed colored from red to blue according to sequence position. The three parts of the ribosome-binding loop are indicated by numbers and orange, green and purple, respectively, and conserved residues interacting with *D. radiodurans* Rpl23p (G42, R44) or *S. cerevisiae* Rpl25p (G22, R24) are in green. Conserved P10 and E14 in Rpl23p and the corresponding P66 and E70 in Rpl25p are in blue. Egd1p and Egd2p are in orange and blue, respectively. K29 of Egd1p, the corresponding R50 of TF, and K30 of Egd1p, pointing to the surface of the ribosome, are in purple. From modeling of Egd1p and Egd2p, we can propose that EGD complex architecture is similar to the TF β-sheet structure although the exact position of α-helices (α1 and α2), indicated by dotted line in Egd1p, and the precise orientation of the Egd1p/Egd2p-interacting domains cannot be determined.

Our experiments show that, in the absence of Egd2p, reduction of Egd1p ubiquitination leads to instability and loss of ribosome binding. It is unclear whether instability might be a consequence of ribosome dissociation or vice versa. To solve this issue, we created an Egd1p mutant in which the conserved ribosome-binding motif $R_{24}R_{25}K_{26}$ was replaced by alanines (Egd1$_{RRK/AAA}$p). This mutant does not associate with the ribosome (WEGRZYN *et al.* 2006) and contains one mutated lysine residue, so we first tested whether it could be ubiquitinated. Indeed, ubiquitination of Egd1$_{RRK/AAA}$p was similar to ubiquitination of wild-type Egd1p (Figure 4E). This mutant was also as stable as wild-type Egd1p in cells lacking Egd2p (Figure 4F, top: compare to Figure 3C, top), indicating that loss of ribosome association *per se* does not lead to Egd1p instability. Interestingly, the deletion of *NOT4*, which leads to reduced Egd1p ubiquitination (Figure 1A and Figure 2D; PANASENKO *et al.* 2006), led to reduced stability of Egd1$_{RRK/AAA}$p in *not4Δ egd2Δ* cells (Figure 4F). Thus, Egd2p and ubiquitination, but not ribosome association, are required for Egd1p stability.

Modeling EGD heterodimer interaction with Rpl25p is compatible with ubiquitination at K29 and/or K30 of Egd1p and possible docking of ubiquitin to the ribosome: Our finding that ubiquitination of Egd1p

was important for its stability and ribosome association was unexpected and led us to investigate the available structural information on the EGD complex, on the one hand, and, on the other hand, on the ribosome binding of a prokaryotic chaperone, TF, which associates with the ribosomal subunit Rpl23p, the homolog of eukaryotic Rpl25p with which Egd1p interacts (SCHLUNZEN *et al.* 2005; WEGRZYN *et al.* 2006). The structures of Rpl23p and Rpl25p present well-conserved folds (SPAHN *et al.* 2004), and the region of Rpl23p interacting with TF (L/IxxPxxS/TExA) is also conserved in all kingdoms of life (WEGRZYN *et al.* 2006). From this observation, one can propose that the ribosome-binding site might be similar between prokaryotic TF and eukaryotic EGD/ NAC. The ribosome-binding domain of TF has a helix-loop-helix fold and interacts with Rpl23p via this loop (KRAMER *et al.* 2002). A similar helix-loop-helix region was found in the predicted secondary structure of Egd1p, whose ribosome-binding loop contains three segments corresponding to the same segments of TF (Figure 5). Despite the reported absence of similarity between TF and Egd1p, we found 38% of sequence identity between the ribosome interacting loop of the *D. radiodurans* TF and the corresponding region of yeast Egd1p (Figure 5). The loop includes both the RRK(x)nKK and GFRxGxxP motifs conserved among eukaryotic and bacterial species,

respectively (KRAMER *et al.* 2002; WEGRZYN *et al.* 2006). In the multiple alignment, G42 and R44 in TF, corresponding to G22 and R24 in Egd1p, are fully conserved. R44 of TF interacts with E14 of Rpl23p (which corresponds to E70 of Rpl25p) and with 23S rRNA (SCHLUNZEN *et al.* 2005). To allow this tripartite interaction, E14 of Rpl23p and R44 of TF have to be ideally placed and oriented, suggesting the unique bound conformation for the ribosome-binding loop of TF. We found that, in spite of the divergence among prokaryotic sequences, predicted secondary structures for the helix-loop-helix region are well conserved. Finally, we noted a strong fold conservation between Rpl23p in prokaryotes and Rpl25p in eukaryotes, in particular in the region interacting with TF or βNAC/Egd1p (Figure 5). Taking into account all of these observations, we can hypothesize that the main requirement for the binding of TF or Egd1p to the ribosome is the ability of the loop to adopt specifically this bound conformation. Thus, we used the ribosome-binding domain of TF as a template and proposed a model for the interaction of the yeast EGD complex with the ribosome.

According to our model, lysines K29 and K30 of Egd1p are the most exposed residues of the loop and could be post-translationally modified (Figure 5). This observation is in good agreement with our data that one or both of these lysines are ubiquitinated. Furthermore, the proximity of K29 and K30 of Egd1p to the two ribosomal proteins Rpl25p and Rpl26Ap (not shown) in our model allows us to suppose that these ribosomal proteins might interact with ubiquitinated Egd1p and stabilize the interaction of the EGD complex with the ribosome. Protein–protein docking experiments of ubiquitin (ligand) against Rpl25p-Rpl26Ap (receptor) produces a collection of solutions, all of which indicate binding of ubiquitin to Rpl25p. In one of the best solutions in terms of electrostatics and shape (total interaction energy of -335 kJ/mol against -377 kJ/mol for the first solution), ubiquitin presents its C terminus to K29 and K30. These findings are in good agreement with our finding that ubiquitination of Egd1p at these residues supports its ribosome association.

Egd2p interacts with proteasome: Recently, it has been shown that the Ccr4-Not complex is associated with the proteasome (LARIBEE *et al.* 2007), and previous studies have described proteasome association of the ubiquitin-conjugating enzymes Ubc4p and Ubc5p in the presence of translationally damaged proteins (CHUANG and MADURA 2005). Taking into account that the EGD complex is thought to be a chaperone protecting newly synthesized polypeptides as they emerge from the ribosome, and that it forms a tertiary complex with Not4p and Ubc4p/Ubc5p (PANASENKO *et al.* 2006), we investigated whether the EGD complex might interact with the proteasome. Using Tap-tagged Rpn1p, we purified different subcomplexes of the proteasome: holoenzyme, RP, and CP. In the purified holoenzymes from both
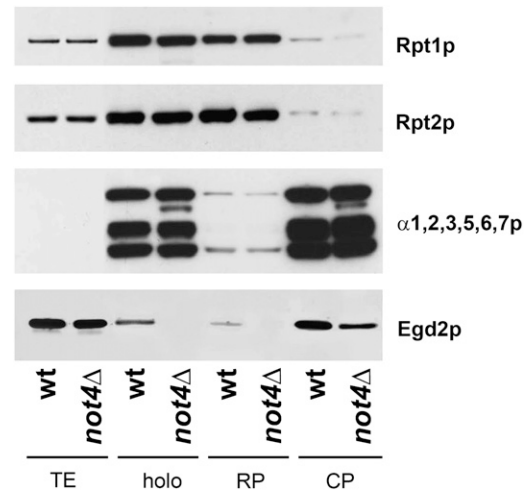

FIGURE 6.—Egd2p interacts with the proteasome. Proteasome holoenzyme (holo), RP, and CP were purified from wild-type or *not4Δ* cell extracts by tandem affinity purification through the Rpn1p subunit. The presence of the indicated proteasome subunits was analyzed in TEs and in the different proteasome fractions by Western blot using antibodies against Rpt1p and Rpt2p or the α-subunits. α1, -2, -3, -5, -6, and 7 levels in TE were detectable only upon longer exposure times and were the same in wild type and *not4Δ* (data not shown). Copurification of Egd2p was analyzed by probing the blots with antibodies against Egd2p.

strains, we found all of the analyzed proteasomal subunits, while in the subcomplexes we found only the subunits specific for these particles, namely for the regulatory subunits Rpt1p and Rpt2p and for the core particles α1, -2, -3, -5, -6, and -7 (Figure 6). Egd2p copurified with the holoenzyme from wild-type but not from *not4Δ* cells, while the level of Egd2p was the same in total extracts from both strains (Figure 6, bottom). We observed an enrichment of Egd2p in the purification of the CP, and again, the levels of copurifying Egd2p were higher from wild-type than from *not4Δ* cells. We conclude that Egd2p can interact with the proteasome, mostly with the CP, and that the deletion of *NOT4* reduces this interaction.

DISCUSSION

Characterization of EGD ubiquitination: Recently, we determined that the Not4p E3 ligase, a subunit of the evolutionary conserved Ccr4-Not complex, could ubiquitinate the EGD complex, called NAC in higher eukaryotes, in a regulated manner (PANASENKO *et al.* 2006). However, the role of EGD ubiquitination *in vivo* is unknown. In this work, we set out to investigate the function of this ubiquitination by first creating and analyzing a series of Egd1p and Egd2p mutants, where the most conserved lysine residues were changed to arginine. For Egd1p, mutation of lysines 29 and 30 to arginine was sufficient to prevent ubiquitination, suggesting that

ubiquitination of Egd1p occurs mainly at lysine residues 29 and/or 30 and might involve the addition of more than one ubiquitin residue at these sites. For Egd2p, ubiquitination was reduced only when a large number of lysine residues were simultaneously mutated to arginine, such as lysines 18, 19, 26, 42, 52, and 70. These results may indicate that in Egd2p multiple sites can be monoubiquitinated.

Interestingly, we observed that ubiquitination of the EGD subunits is linked. It seems that Egd1p is the main ubiquitinated subunit, but that Egd2p can be ubiquitinated either when Egd1p is unable to be ubiquitinated or in response to an environmental change.

We made the surprising observation that deletion of Ubc5p, but not deletion of Ubc4p, both E2 partners of the Not4 E3 ligase, leads to an increase in Egd1p ubiquitination, and at the same time that Ubc5p, but not Ubc4p, is an unstable protein. It could be that Ubc5p is more readily recruited to ubiquitinate Egd1p with Not4p, but is not the most efficient E2 for Egd1p ubiquitination. In its absence, Not4p would then work with another E2, possibly Ubc4p, more efficiently in Egd1p ubiquitination. In this regard, it is interesting to note that we observed an increase in Ubc4p levels in cells lacking Ubc5p (data not shown). Thus, a reduction of *de novo* Ubc5p synthesis is one way to obtain more efficient Egd1p ubiquitination, for example, in response to environmental clues.

The UBA domain of Egd2p is important for EGD complex stability: The structure of aeNAC has revealed that it is a homodimer that associates through the NAC domains, indicating that eukaryotic α- and βNAC subunits are likely to dimerize via their NAC domains. This idea was supported by homology modeling using the homodimeric NAC domain of aeNAC as a template for the yeast and human heterodimeric NAC domains as shown in previous work (SPRETER *et al.* 2005) and supported in our own study. Egd2p is stable in yeast cells from which Egd1p has been depleted (PANASENKO *et al.* 2006), but is no longer associated with ribosomes (REIMANN *et al.* 1999). In contrast, Egd1p is unstable in yeast in the absence of Egd2p, even though it can form homodimers *in vivo* (PANASENKO *et al.* 2006). An obvious question is, since Egd1p can exist in the form of homodimers, why is it unstable in the absence of Egd2p? In other words, what is specific in the structure of Egd2p that stabilizes its partner? Egd2p contains a UBA domain, and this has interesting implications. UBA domains are found in very diverse proteins involved in protein degradation, cell cycle control, and DNA repair and have been shown to bind ubiquitin or polyubiquitin. NMR studies have revealed that UBA domains interact via a hydrophobic patch within ubiquitin (MUELLER *et al.* 2004). Such a solvent-exposed hydrophobic patch is also present on the aeNAC UBA domain, and the hydrophobic residues are conserved in all NAC homologs (SPRETER *et al.* 2005). In the absence

of its UBA domain, Egd2p is very unstable (PANASENKO *et al.* 2006), although the truncated protein is also expected to be able to homodimerize because the UBA domain is structurally separate from the NAC domain (SPRETER *et al.* 2005). This protein can be stabilized if it is fused to an N-terminal tag such as GFP or B42-HA (PANASENKO *et al.* 2006). Nevertheless, even if yeast cells express stable UBA-less Egd2p, Egd1p, particularly non-ubiquitinated Egd1p, is unstable. This observation suggests that the UBA domain of Egd2p is involved in Egd1p stabilization directly, and not only via stabilization of Egd2p itself.

Recently, it was found that in the fission yeast *Schizosaccharomyces pombe* αNAC did not interact with monoubiquitin or with K48-linked ubiquitin chains *in vitro* (ANDERSEN *et al.* 2007). The same results were obtained earlier for the baker's yeast Egd2p UBA domain (RAASI *et al.* 2005). All these data were obtained *in vitro*, so it remains possible that the UBA domain of Egd2p can interact with ubiquitinated Egd1p or maybe with ubiquitinated Egd2p itself, within the EGD complex, whose structure might favor such an interaction. Heterodimerization of Egd1p and Egd2p might subsequently protect the complex from degradation. The surprise was the finding that the UBA domain of Egd2p is particularly important for Egd1p stability if Egd1p is not ubiquitinated. However, we observed that, in this case, ubiquitination of Egd2p increases, but much less so in the absence of the UBA domain. It is possible that the Egd2p UBA domain can bind either ubiquitinated Egd1p or Egd2p (if and when Egd1p is not ubiquitinated) to stabilize ubiquitination and thus the EGD complex.

The demonstration of the importance of the Egd2p UBA domain for the stability of the EGD complex is exciting. Indeed, while a number of ubiquitin-binding domains have now been described, there are still few examples of the role of these domains *in vivo*. The UBL/UBA domain protein Rad23p contributes to shuttling polyubiquitinated substrates to the proteasome, and the Rad23p UBA domains might compete with the proteasome for these substrates, thereby inhibiting ubiquitin-mediated degradation and stabilizing proteins (RAASI and PICKART 2003). Such a protective role for UBA domains has been shown for several other yeast proteins, namely Dsk2p, Ddi1p, and Ede1p (HEESSEN *et al.* 2005). Our work is clearly in line with these published results, except that it additionally suggests that the Egd2p UBA domain is required not only for Egd2p stability, but also for the stability of its interacting partner, Egd1p.

Ubiquitination and Egd2p support Egd1p association with the ribosome: EGD was originally characterized as the first ribosome-associated protein to contact the emerging, not yet properly folded, polypeptide chains and to contribute to fidelity in cotranslational processes such as targeting and folding (WIEDMANN

et al. 1994). Only the Egd1p subunit contacts the ribosome directly, while both subunits of EGD interact with newly synthesized polypeptide chains (Wiedmann *et al.* 1994; Beatrix *et al.* 2000). In this work, we found that efficient Egd1p ribosome association is dependent upon Not4p, Egd2p, and its own ubiquitination. These results were considered in light of a modeling of the EGD heterodimer interaction with ribosomal protein Rpl25p. According to our model, the N terminus of Egd1p is in direct contact with Rpl25p in agreement with earlier data (Beatrix *et al.* 2000). However, Egd2p contributes with Egd1p to form a conformation suitable for ribosome binding, which might be similar to the described association of prokaryotic TF with the ribosome (Kramer *et al.* 2002). Our experimental data revealing that Rpl25p is co-immunoprecipitated with non-ubiquitinated Egd1p less efficiently in the absence of Egd2p are in good agreement with this model. Concerning the role of ubiquitination, our model suggests that lysines K29 and K30 are the most exposed residues of the ribosome-binding loop of Egd1p, and therefore they could indeed be ubiquitinated. Moreover, interactions between Rpl25p and ubiquitin are likely to occur if ubiquitin is C-terminally linked to either K29 or K30 and might stabilize the interaction of the EGD complex with the ribosome.

Obviously, our results are intriguing in light of the knowledge that EGD can bind Rpl25p *in vitro* in the absence of any ubiquitination (Wegrzyn *et al.* 2006), and, furthermore, that in cell extracts only a small portion of Egd1p seems to be ubiquitinated, yet binding of EGD to the ribosome has been reported to be stoichiometric. While we have no definitive explanations to answer these questions, it is likely that, *in vivo*, binding to the ribosome is more complex and competitive than in the experiments performed *in vitro*, where only the two binding partners were put together, and thus it is not unreasonable to suggest that additional mechanisms contribute to binding *in vivo*. Concerning the efficient binding to ribosomes *in vivo* and the relatively small percentage of visible ubiquitinated Egd1p in total extracts, one can imagine that the contribution of ubiquitination is transient upon initial binding yet is not needed subsequently. But it is also likely that Egd1p *in vivo* is ubiquitinated to higher extents than visible in total extracts and simply becomes rapidly deubiquitinated upon cell lysis. Indeed, it is generally the case that ubiquitinated proteins are modified only to a reduced degree when analyzed in cell extracts. We observed that although EGD ubiquitination increases as cells grow from exponential phase to saturation, no difference in ribosome association could be measured (our unpublished results), revealing, not surprisingly, that ubiquitination is not the only determining factor for the level of EGD ribosome association. In this context, we found that the mutation of lysines 10, 13, 29, and 30 to positively charged arginine

had only a weak effect on Egd1p ribosome association when Egd2p was still present, while former work (Wegrzyn *et al.* 2006) showed that the mutation K29,30A disrupted ribosome association. This leads us to suggest that, in addition, a positive charge in this region of Egd1p might contribute to ribosome binding.

In any event, taken together, our results lead us to suppose that there are two factors important for interaction of EGD with the ribosome: ubiquitination of the ribosome-binding loop of Egd1p and Egd2p. The role of Egd2p may be related to its capacity to be ubiquitinated if Egd1p is not, since the ribosome association of Egd1p is disrupted in cells expressing mutants of Egd1p and Egd2p that both have reduced ubiquitination or in cells lacking Not4p where ubiquitination of both Egd1p and Egd2p is lost.

The importance of ubiquitination for EGD ribosome association is interesting. Indeed, originally linked to protein degradation by the proteasome, it is now clear that, in cells, ubiquitination serves as a reversible modification, similarly to phosphorylation, by the concerted action of enzymes that place and remove the modification. Many functions have been attributed to ubiquitination. Indeed, ubiquitin is a particularly versatile modification because of its ability to modify proteins in a monomeric form or to be conjugated to preceding ubiquitin moieties and thus form many types of ubiquitin chains, thereby creating a great variety of molecular signals in the cell (for review, see Ikeda and Dikic 2008). A large number of ubiquitin-binding domains have been identified, some of which bind preferentially to ubiquitin chains with a linkage specificity, whereas others are more promiscuous. The binding affinity between ubiquitin-binding domains and ubiquitin is generally relatively low for a physiological interaction, suggesting that physiological interactions between ubiquitinated substrates and ubiquitin-binding proteins are likely to be mediated by multivalent interactions. This might be the case for the interaction revealed in this study since EGD is able to bind Rpl25p *in vitro* in the absence of any ubiquitination, which becomes relevant *in vivo*. Understanding exactly how ubiquitination of both Egd1p and Egd2p contribute to this binding *in vivo* is certainly an exciting challenge for future studies.

A function for EGD linking the ribosome and the proteasome: Our study also revealed an interaction between Egd2p and the proteasome. This finding can be considered in light of the demonstrated cotranslational ubiquitination (Schubert *et al.* 2000; Turner and Varshavsky 2000), which implies the existence of a ribosome-associated ubiquitin ligase. Ubiquitination of EGD maybe connected in some way to ubiquitination of newly synthesized polypeptide chains on the ribosome or to the transport of these chains to the proteasome. In this context, first, Not4p, which is an E3 ubiquitin ligase that interacts with EGD and can ubiquitinate it *in vitro*, has been shown to interact with the proteasome

(Laribee *et al.* 2007). Second, Ubc4p, which works as an E2 enzyme together with Not4p to ubiquitinate EGD, has also been shown to associate with the proteasome in response to translationally damaged proteins (Chuang and Madura 2005). Thus one can imagine a possible new function of EGD, which involves connecting events occurring at the ribosome with the proteasome. Maybe Egd2p can be recruited to the proteasome via its UBA domain, as in the case of Rad23p (Raasi and Pickart 2003). Interestingly also, the efficient interaction of Egd2p with the proteasome required Not4p and probably therefore the Ccr4-Not complex. Clearly understanding the coordination of protein synthesis with protein degradation, and the role of the EGD and Ccr4-Not complexes in this coordination, opens intriguing new areas of investigation in the future.

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