Distinct Steps in Dislocation of Luminal Endoplasmic Reticulum-associated Degradation Substrates

**ROLES OF ENDOPLASMIC RETICULUM-BOUND p97/Cdc48p AND PROTEASOME**

Received for publication, September 8, 2003, and in revised form, November 3, 2003
Published, JBC Papers in Press, November 8, 2003, DOI 10.1074/jbc.M309938200

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Dislocation of endoplasmic reticulum-associated degradation (ERAD) substrates from the endoplasmic reticulum (ER) lumen to cytosol is considered to occur in a single step that is tightly coupled to proteasomal degradation. Here we show that dislocation of luminal ERAD substrates occurs in two distinct consecutive steps. The first is passage across ER membrane to the ER cytosolic face, where substrates can accumulate as ubiquitin conjugates. In vivo, this step occurs despite proteasome inhibition but requires p97/Cdc48p because substrates remain entrapped in ER lumen and are prevented from ubiquitination in cdc48 yeast strain. The second dislocation step is the release of accumulated substrates to the cytosol. In vitro, this release requires active proteasome, consumes ATP, and relies on salt-removable ER-bound components, among them the ER-bound p97 and ER-bound proteasome, which specifically interact with the cytosol-facing substrates. An additional role for Cdc48p subsequent to ubiquitination is revealed in the cdc48 strain at permissive temperature, consistent with our finding that p97 recognizes luminal ERAD substrates through mult ubiquitin. Bip interacts exclusively with ERAD substrates, suggesting a role for this chaperone in ERAD. We propose a model that assigns the cytosolic face of the ER as a midpoint to which luminal ERAD substrates emerge and p97/Cdc48p and the proteasome are recruited. Although p97/Cdc48p plays a dual role in dislocation and is involved both in passage of the substrate across ER membrane and subsequent to its ubiquitination, the proteasome takes part in the release of the substrate from the ER face to the cytosol en route to degradation.

The endoplasmic reticulum-associated degradation (ERAD) is a quality control process that selectively eliminates malformed proteins or unassembled subunits of oligomeric proteins in the secretory pathway (1, 2). ERAD substrates are dislocated from the endoplasmic reticulum (ER) back to the cytosol via the Sec61 complex (3–6). In the cytosol, ubiquitin is conjugated to the ERAD substrates that are degraded by the proteasome (7). The proteolytically active proteasome has been implicated in the dislocation of ERAD substrates from the endoplasmic reticulum (ER) to the cytosol of proteasome-inhibited cells (8–11). Although stabilization of proteins in the secretory pathway by proteasome inhibitors is generally accepted as an indication for ERAD, ubiquitination of such proteins is a direct evidence for the access of the substrate to the cytosol (7). In most cases, however, accumulation of the multiubiquitinated ERAD substrates to detectable levels requires inhibition of the proteasome. If the proteasome is indeed required for dislocation (8–11), then ubiquitination of ERAD substrates would not be observed. This applies especially to luminal ERAD substrates, as membrane ERAD substrates inherently display cytosolic domains, which may be ubiquitinated irrespective of dislocation (11–15). Therefore, it is our view that luminal ERAD substrates, whose dislocation is an absolute prerequisite for ubiquitination, are the proteins of choice to address directly coupling among dislocation, ubiquitination, and proteasomal activity.

Among luminal ERAD substrates, the best characterized are CPY* in yeast (5, 16, 17) and immunoglobulins (Igs) in mamalian cells (8, 9). Igs are particularly attractive because they are oligomeric proteins in the secretory pathway composed of heavy and light chains, both of which are subjected to proteasomal degradations. This is indicated by the stabilization of μs chains, the heavy chains of secretory IgM (9, 18, 19) or of Igκ light chains (8), upon proteasome inhibition. Under these conditions, μs or κ accumulate in the microsomal fractions rather than the cytosol, implicating the proteasome in dislocation (8, 9). Nevertheless, we detected ubiquitination of the luminal μs when the proteasome proteolytic activity was inhibited (20), indicating that the proteasome was not required for μs to gain access to the cytosol-facing ubiquitination machinery.

Using cell fractionation, protease protection assay, and salt wash in B lymphocytes and yeast, as well as in vitro dislocation experiments, here we dissect dislocation of luminal substrates into two consecutive steps: (i) passage across the ER membrane and (ii) release to the cytosol. We investigate the roles of the proteasome and of the recently discovered cytosolic ERAD chaperone p97/Cdc48p (21) in dislocation and reveal differential requirements for these components in the two dislocation steps. We define the cytosolic face of the ER membrane as a site to which luminal ERAD substrates are fully dislocated and cytosolic ERAD components are recruited, extending the ER-bound ERAD machinery (7) to include the ER-bound subpopulation of the proteasome (22, 23) as well as the ER-bound subpopulation of p97/Cdc48p.

This paper is available on line at http://www.jbc.org
EXPERIMENTAL PROCEDURES

Mammalian Cell Lines, Yeast Strains, Plasmids, Transfection, and Transient Expression—As described previously (24), COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (In-vitrogen) supplemented with 10% fetal calf serum and penicillin/streptomycin/nystatin (3.75 units/ml). COS-7 cells were transfected according to the calcium phosphate method (25), and cells were assayed 48 h post-transfection. For μ expression, genomic pJC119-cep (a kind gift from Y. Argon, University of Chicago) was used. For myc-tagged wild-type or K48R ubiquitin expression, we used described plasmids (12) (a kind gift from R. Kopito, Stanford University). Because among all of the cdc48 alleles, which exhibited similar defects in ERAD (cdc48-1 (our results) (21) as well as (16, 26), cdc48-2(27), cdc48-3(26), cdc48-5(28, 29), cdc48-7(2), cdc48-10 (21) and cdc48-11 (16)), the temperature-sensitive strain A () was highly reproducible for in vivo, we naturally chose to advance our study with this strain and its isogenic strain KFY100, which expresses wild-type CDC48 for comparison. The HA-tagged CPY* (pre-t-1 allele) was expressed from plasmid pBG15 (generously provided by A. A Cooper, University of Missouri-Kansas City).

Bioisotopic Labeling and Cell Incubation—Cells were starved for methionine for 45 min and either labeled for 45 min or pulse labeled for 15 min with [35S]methionine (100 μCi/ml; 1,000 Ci/mmol) and then chased in the presence of 25 μM ALLN, 5 μM MG-132, or their combinations. When not labeled, cells were incubated for 3–4 h with ALLN, MG-132, or their combinations. Cells and medium were prepared as described previously (17). Yeast cells were washed by resuspension in ice followed by centrifugation (10,000 × g, 20 min, twice) from disrupted cells. PNS was further fractionated by centrifugation at 4°C. B cells were fractionated into P10 (10,000 × g, 30 min) and P200 (200,000 × g, 1 h) microsomal pellets and S200 (200,000 × g, 1 h) cytosol supernatant; yeast cells were fractionated into P20 (20,000 × g, 30 min) microsomal pellets and S20 cytosol supernatant.

For in vitro dislocation assays, 38C cells metabolically labeled for 45 min or unlabeled cells incubated for 3 h with or without various drugs were disrupted in 0.25 M sucrose and 10 mM potassium phosphate, pH 7.5. P10 microsomes were resuspended either in S10 cytosol from equivalent amounts of 38C or COS-7 cells (60 and 30 mg/ml protein, respectively), or in the disruption buffer, all containing 2 mM phenylmethanesulfonyl fluoride and 2 mM kallikrein inhibitor unit/ml apotinin. Resuspended microsomes, supplemented with an ATP-regenerating system (3 mM ATP, 5 units/ml creatine kinase, 10 mM creatine phosphate, 3 mM MgCl2) or treated with 25 units/ml apyrase were incubated at 38°C cells incubated for 4 h at permissive (30°C) or nonpermissive (37°C) temperatures. Cytosol and medium were separated, and cells were assayed 48 h post-transfection. For μ expression, genomic pJC119 (generously provided by A. A Cooper, University of Missouri-Kansas City).

Immunoprecipitation and Immunodetection—IgM μ heavy chains, HA-tagged CPA*, BiP, or myc-tagged ubiquitin and their associated proteins were immunoprecipitated in each experiment from comparable amounts of lysed cells or fractions. For cytosolic fractions, an equal volume of lysate buffer was added. Antibodies used for immunoprecipitation: goat anti-mouse μ (SouthernBiotech), mouse anti-HA (clone 12C5), mouse anti-myc (clone 9E10), were all followed by protein A-Sepharose (Repligen), rabbit anti-mouse BiP (Affinity BioReagents) was followed by goat anti-rabbit IgG-Sepharose (Sigma). Total cell extract proteins and immunoprecipitated μ, HA-CPA*, BiP, or myc ubiquitin were resolved by reducing or nonreducing SDS-PAGE, electroblotted onto nitrocellulose, and detected either by direct autoradiography or by immunoblotting. Antibodies used for immunoblotting were: horseradish peroxidase-conjugated anti-mouse μ (SouthernBiotech); biotin-conjugated mouse anti-BiP (SouthernBiotech) followed by horseradish peroxidase-conjugated avidin (Jackson); rabbit anti-μ (21) and rabbit anti-proteasome α subunit of Methanosarcina thermophila (Calbiochem) followed by horseradish peroxidase-conjugated anti-rabbit IgG (Jackson); mouse anti-μ (clone 12C5A), mouse anti-CPA* (clone 58.13.3; PelFreeze) or mouse anti-ubiquitin (clone P4D1, BabCO) used to detect ubiquitin in B cells and mouse anti-ubiquitin (clone Ubi-1, Zymed Laboratories Inc.) used to detect ubiquitin in yeast, followed by horseradish peroxidase-conjugated anti-mouse IgG (Jackson). The horseradish peroxidase was visualized by the enhanced chemiluminescence (ECL) reaction. The amounts of radiolabeled or immunoblotted proteins were determined by densitometry, and relative levels were calculated.

RESULTS

The luminal μ heavy chain exhibits developmentally regulated stability; while being a stable protein that is secreted efficiently from plasma cells, it is degraded rapidly in the earlier differentiation stages of pre-B and B cells (19, 24, 30). Based on its stabilization by a variety of proteasome inhibitors, we and others have shown that μ is degraded by the proteasome (9, 18, 19). Moreover, in B lymphocytes treated with proteasome inhibitors, we were able to detect ubiquitinized species of μ (Fig. 1A; see also Ref. 20). Therefore, we reasoned that, despite the inhibition of the proteasome, a part of the luminal μ must be dissociated to gain access to the cytosol-facing ubiquitination machinery. To test this hypothesis, we fractionated IgM-expressing 38C B cells and determined the topology of μ upon proteasome inhibition.

Enhanced BiP Association with μs upon Proteasome Inhibition Suggests a Role for BiP in μs Dislocation—Upon fractionation of 38C B cells, the majority (~80%) of μs was detected in the microsomal fraction, whereas only ~20% was recovered in the cytosolic fraction (Fig. 1A, A and D). The BiP that coprecipitated with μs was negligible and restricted to the microsomal fraction (Fig. 1A, lanes 1 and 2). Upon blocking the proteasome, there was a ~60% increase in the cellular content of μs, accompanied by 5–7-fold increase in the amount of coprecipitated BiP (Fig. 1A, lanes 1, 5, and 9; Fig. 1G). This increase in μs was not recovered in the cytosolic fraction but rather in the microsomal fraction (Fig. 1A, A and D). The enhanced coprecipitation of BiP could not be explained by accumulation of un-assembled μ heavy chains, the favored substrate of BiP (31), because none was detected (Fig. 1B, upper panel, lanes 1 and 2). Importantly, this increase was not accompanied by any rise in cellular BiP content (Fig. 1C, compare lanes 5 and 9 with lane 1), indicating that the unfolded protein response was not elicited. Also, in reciprocal experiments, increased amounts of μs coprecipitated with an anti-BiP antibody (Fig. 1B, compare lanes 4 and 5 in the upper panel and lanes 5 and 6 in the lower panel). Thus, in proteasome-inhibited cells the physical stability of the BiP-μs complexes is enhanced. Our findings concur with the previously reported enhanced BiP coprecipitation and increased physical stability of the BiP-κ light chain complexes in proteasome-inhibited NCS1 cells (8). Interestingly, BiP pulled down μs assemblomeed with κ into μsκ monomers but not unassembled μs (Fig. 1B, upper panel, lanes 4 and 5). Furthermore, BiP pulled down only the unstable ERAD substrate μ but not its stable membrane isoform μ (Fig. 1B, compare lanes 5 and 6 with lanes 1 and 2 in the lower panel and lanes 4 and 5 with lanes 1 and 2 in the upper panel). Taken together, our results suggest a novel role for BiP in ERAD of an already assembled secretory IgM, in addition to its established role in Ig assembly.

E. Rabinovich and S. Bar-Nun, unpublished results.
FIG. 1. Ubiquitinated χ accumulates at the cytosolic face of ER in association with microsome-bound proteasome and p97. 38C cells incubated with ALLN, MG-132 (MG) or none were disrupted, PNS was treated with (+) or without (−) trypsin, fractionated, and P10 (P) and S200 (S) fractions were lysed. Total lysates (20%; C) or immunoprecipitates (IP) obtained with anti-μ antibody from 80% of lysates (A) were resolved by reducing SDS-PAGE. Electroblotted proteins were probed (IB) and reprobed with the indicated antibodies: anti-μ, anti-BiP, anti-ubiquitin (anti-Ub), anti-p97, and anti-proteasome α subunit (anti-α). B, immunoprecipitates were obtained in parallel with anti-μ or anti-BiP from lysed cells or lysed fractions and were resolved by nonreducing (upper panel) or reducing (lower panel) SDS-PAGE. Note that anti-rabbit IgG-Sepharose without anti-BiP cannot coprecipitate any χ (upper panel, lane 3). D, μs (A, upper panel) was quantified by densitometry in the various fractions (cytosolic, luminal (trypsin-resistant) or dislocated (trypsin-sensitive)) and calculated as the percent of its sum (100%) in the various fractions in each treatment (none, ALLN, MG-132). The data presented are the average from three independent experiments. E, microsomal μs was calculated as the percent of its level in microsomes from cells incubated with no inhibitors which were not treated with trypsin (100%). The data presented are the average from three independent experiments. F, P10 microsomes were resuspended in 0.5 M KCl and wash supernatant, and washed microsomes were separated by centrifugation. Proteins were resolved by reducing SDS-PAGE, electroblotted and probed with anti-μ antibody (upper panel), and reprobed with an anti-BiP antibody (lower panel). G, BiP, proteasome α subunit, and p97 coprecipitated with μ from microsomes obtained from untreated (none) or proteasome-inhibited cells (ALLN, MG-132), with (+) or without (−) trypsin treatment, were quantified by densitometry. The values are presented as fold increase, with values in untreated cells being set as 1. The data presented are the average from three independent experiments. μ, free μ heavy chain; μs, hemimers; μs×2, monomers; Ub-μ, ubiquitinated μ.
In agreement with the genetic data in yeast (32), our results provide the first biochemical evidence for involvement of BiP in ERAD of luminal substrates in mammalian cells.

Luminal \( \mu s \) Crosses the ER Membrane and Accumulates at the ER Cytosolic Face Despite Proteasome Inhibition—The finding that \( \mu s \) undergoes ubiquitination (20) prompted us to examine the distribution of ubiquitinated \( \mu s \) in fractionated, proteasome-inhibited B cells. Heavily ubiquitinated \( \mu s \) was observed mostly in microsomes, in correlation with the distribution of the \( \mu \) protein (Fig. 1A). These results demonstrated that ubiquitinated \( \mu s \) was associated with the microsomes while facing the cytosol.

Protease protection assays demonstrated that the cytosol-facing ubiquitinated \( \mu s \) represented a significant proportion of the \( \mu \) protein. In untreated cells, only \( \sim 8\% \) of microsomal \( \mu s \) was sensitive to trypsin, whereas in proteasome-inhibited cells, where microsomal \( \mu s \) rose to \( \sim 160\% \), more than half of it was digested by trypsin (Fig. 1A and quantified results in Fig. 1E). We conclude that upon proteasome inhibition microsomal \( \mu s \) was redistributed between the two sides of the membrane so that the dislocated \( \mu s \) increased by 7-fold, whereas cytosolic \( \mu s \) hardly increased (Fig. 1D). As expected, all \( \mu s \) ubiquitin conjugates were completely digested by trypsin (Fig. 1A). Importantly, the integrity of microsomal membranes was demonstrated by the complete protection of BiP from trypsin digestion (Fig. 1, A and C). The complete digestion of either dislocated \( \mu s \) (Fig. 1A) or luminal \( \mu s \) in Nonidet P-40-solubilized microsomes (data not shown) indicated that \( \mu s \) could be digested by trypsin. An additional indication for the membrane integrity was the fate of \( \mu \), the stable membrane isoform of \( \mu \) heavy chains (24). Apart from the KVK sequence in its C terminus, which faces the cytosol, and its single transmembrane span, the rest of this protein is luminal oriented. Indeed, \( \mu \) was detected exclusively in microsomes and was fully protected from trypsin digestion regardless of proteasome inhibition (Fig. 1A).

Another evidence for the cytosolic orientation of microsomal \( \mu s \) was afforded by the ability to remove it from the microsomal fractions by salt wash. When the proteasome was inhibited, the amounts of \( \mu s \) removed by salt increased considerably (Fig. 1F, lanes 1 and 2), compared with the moderate increase in the amounts of \( \mu s \) which remained in the washed microsomes (Fig. 1F, lanes 3 and 4). Importantly, neither \( \mu \) nor luminal BiP was removed by salt wash, and both remained with the washed microsomes (Fig. 1F, lower panel, lanes 3 and 4). Taken together, our findings define the cytosolic face of the ER membrane as a site to which the luminal ERAD substrate \( \mu s \) is fully dislocated. Not only the arrival of \( \mu s \) to this site occurs despite proteasome inhibition, but also the latter is a prerequisite for revealing this site.

Dislocated \( \mu s \) Interacts Exclusively with the Microsome-bound Proteasome and Microsome-bound p97—Although the active proteasome appeared to play no role in \( \mu s \) dislocation, coprecipitation of the proteasome with \( \mu \) indicated their physical interaction, which was actually enhanced ~2.5-fold when the proteasome was proteolytically inactive (Fig. 1A, lanes 1, 5, and 9; Fig. 1G). Remarkably, this interaction with \( \mu \) was restricted to the proteasome subpopulation that was membrane-bound (Fig. 1A, lanes 1, 5, and 9). This finding suggests a role for the previously identified small ER-bound subpopulation of this cytosolically abundant ERAD component (22) and demonstrates, for the first time, interactions that involve this subpopulation. Moreover, these results are in agreement with the suggestion that the proteasome might be recruited to the ER membrane by mult ubiquitinated ERAD substrates (23).

Recent findings have implicated the p97/Cdc48p as a novel cytosolic component essential for ERAD (16, 21, 26–28), and we have shown that p97 physically interacts with \( \mu \) heavy chains (21). Therefore, we investigated further the interaction of p97 with \( \mu s \) in fractionated cells. Evidently, the ~20% cytosolic \( \mu s \) (Fig. 1D) failed to coprecipitate p97 (Fig. 1A), although the pool of p97 was mostly cytosolic (90%; Fig. 1C, lanes 1 and 2). Conversely, p97 coprecipitated with \( \mu s \) only in the microsomal fraction (Fig. 1A). Assuming that the observed interaction of p97 with \( \mu s \) was direct, it was remarkable that as little as 6.5% of already dislocated \( \mu s \) could efficiently precipitate a large proportion of the minute subpopulation of microsome-bound p97 (Fig. 1A). Proteasome inhibition enhanced ~2.5-fold the amounts of microsome-bound (Fig. 1C) as well as coprecipitated p97 (Fig. 1, A and G), whereas the p97 cytosolic form increased only marginally (Fig. 1C). As expected, p97 and the proteasome, which interacted with the cytosol-facing ubiquitinated microsomal \( \mu s \), were also sensitive to trypsin, whereas BiP, which interacted with the luminal \( \mu s \), was not (Fig. 1, A and G). We conclude that from the abundance of the proteasome and p97 found in the cytosol, only the minor membrane-bound subpopulations of these components are engaged with the ERAD substrate \( \mu s \).

Dislocation of Luminal Substrates Involves p97/Cdc48p—To test directly the suggested role of the essential protein p97/Cdc48p in dislocation (16, 21, 26), we took advantage of yeast genetics, relying on the remarkable conservation of the ERAD pathway from yeast to mammalian cells. We chose the yeast strain carrying the temperature-sensitive cdc48-10 allele, for which we have demonstrated previously the marked stabilization of ERAD substrates at 37 °C (21). Here, we biochemically monitored the distribution of the luminal ERAD substrate HA-CP* with respect to the membrane of the microsomes. The majority of microsomal HA-CP*, a luminal ERAD substrate in yeast, was protected from trypsin digestion when cells were grown at 37 °C but not at 30 °C (Fig. 2A, lanes 1, 2, 4, and 5). In the wild-type CDC48 strain, similar levels of HA-CP* were protected at both temperatures (Fig. 2A, upper panel). These results implicate Cdc48p in the dislocation process. Interestingly, in the mutant cdc48 at the permissive temperature, higher levels of HA-CP* were trypsin-sensitive, compared with wild-type CDC48 (Fig. 2A). This suggested that events following dislocation were relatively slowed down in the mutant, resulting in substrate accumulation at the ER cytosolic face. The lingering of microsomal HA-CP* protein in the lumen was corroborated further by salt wash experiments in mutant cdc48 cells that were shifted from 30 °C to 37 °C. There, the amounts of HA-CP* removed by salt wash decreased significantly (Fig. 2B), in agreement with the decreased susceptibility of this luminal ERAD substrate to digestion by trypsin (Fig. 2A).

p97/Cdc48p Acts before Mult ubiquitination but Also Recognizes Substrates through Their Mult ubiquititin Moieties—The interaction of p97 with dislocated microsomal \( \mu s \), which was enhanced upon proteasome inhibition and accumulation of mult ubiquitinated \( \mu s \) (Fig. 1, A and G), raised the possibility that p97 recognized the luminal ERAD substrate \( \mu s \) through its attached mult ubiquitin moieties. This is an attractive possibility because p97 directly interacted with mult ubiquitin (29, 33), and mult ubiquitination was implicated previously in dislocation of several ERAD substrates (14, 16). To examine in vivo the effect of mult ubiquitination on the physical interactions between \( \mu s \) and p97, we took advantage of the COS-7-based transient expression system. First, we show that the fate of \( \mu s \) in COS-7 cells was consistent with our findings in B cells because in both cell lines \( \mu s \) was rapidly degraded, and this degradation was blocked by proteasome inhibitors (Fig. 3A; see also Ref. 20). Next, wild-type or mutant (K48R) myc-tagged...
ubiquitin was overexpressed in naïve or μs-expressing COS-7 cells. To avoid a high background of ubiquitin signal, the following experiments were carried out in the absence of proteasome inhibitors. As shown in Fig. 3B, overexpression of wild-type ubiquitin resulted in abundance of myc-tagged ubiquitin-conjugated proteins, which were immunoprecipitated by anti-myc and probed with anti-ubiquitin antibodies (lane 6). The enhanced ubiquitination was also reflected by the slight increase in ubiquitin-conjugated μs, which was immunoprecipitated by anti-μ and probed with anti-ubiquitin antibodies (compare lanes 3 and 9). Overexpression of K48R abolished the enhanced levels of myc-tagged multiubiquitinated proteins (compare lanes 6 and 8), as reflected also by dumping the slight increase in μs ubiquitination (compare lanes 9 and 11). Consequently, μs was stabilized (compare μs in lanes 9 and 11), even if only marginally, similar to cystic fibrosis transmembrane conductance regulator (12). Next, we correlated the degree of ubiquitination to the binding of p97. The specific interaction of p97 with ERAD substrates was recapitulated when p97 was coprecipitated by an anti-μ antibody in μs-expressing but not in mock-transfected COS-7 cells (compare lanes 1 and 3). Moreover, we reciprocated in vivo the in vitro findings of Dai and Li (33) by showing that regardless of μs expression, p97 was pulled down by the abundant myc-tagged multiubiquitinated proteins (lane 6), but not by proteins decorated with myc-tagged K48R (lane 8). This demonstrated that the latter population, which was immunoprecipitated with an anti-myc antibody and therefore contained only proteins with impaired multiubiquitination, indeed could not bind p97.

Surprisingly, in μs-expressing COS-7 cells, the interaction between μs and p97 (monitored by the levels of p97 that were pulled down by an anti-μ antibody) was abolished by overexpression of wild-type ubiquitin but was restored by overexpression of K48R ubiquitin (Fig. 3B, compare lanes 3, 9, and 11). We interpreted these data as an indication for competition between the abundance of myc-tagged multiubiquitinated proteins and the multiubiquitinated μs for binding to p97. Competition of multiubiquitin chains with cellular ubiquitinated proteins for...
binding to p97 was reported previously (33). On the other hand, proteins in K48R-expressing cells with impaired multiubiquitination failed to compete with p97 because they bind p97 poorly. In these cells, the diminished coprecipitation of p97 by the anti-myc antibody, but not by the anti-\(\mu\) antibody, probably reflected the mixed population of \(\mu\)s, carrying not only short K48R-containing ubiquitin chains but also normal high molecular weight multiubiquitin moieties that bind p97 efficiently. Taken together, p97 appears to recognize the luminal ERAD substrate \(\mu\)s through the same mechanism by which it recognizes many proteasomal substrates, namely through their multiubiquitin moieties.

In light of our observation that p97 recognized \(\mu\)s through its multiubiquitin moiety, together with the suggested role of multiubiquitination in dislocation of ERAD substrates (14, 16) we sought to pinpoint the function of Cdc48p with respect to multiubiquitination. Again, by using the \(cde48-10\) allele, immunoprecipitation of HA-CPY* with an anti-HA antibody followed by probing with an anti-ubiquitin antibody revealed that the substantial ubiquitination of HA-CPY* at 30 °C was completely abolished at 37 °C (Fig. 2C, lanes 3 and 4). Together with the nearly complete protection from trypsin digestion observed at 37 °C, this further indicated the involvement of Cdc48p in dislocation. Interestingly, in the \(CDC48\) wild-type strain, a
Roles of p97/Cdc48p and Proteasome in ERAD

A. S10 supernatant

B. Buffer supernatant

C. Supernatant

Fig. 4. In vitro reconstitution of $\mu$s dislocation relies on microsome-bound components. P10 microsomes from 38C cells were incubated for the indicated time with either S10 cytosol or buffer, and the released $\mu$s was recovered from the supernatant. As indicated, S10 or buffer was untreated (none) or treated with apyrase or supplemented with ATP. A, microsomes obtained from cells labeled for 1 h with $^{[35]}$S-methionine were incubated with S10 from unlabeled 38C cells, released $\mu$s was immunoprecipitated (IP), resolved by reducing SDS-PAGE, electrophoretically transferred to nitrocellulose, and reprobed with anti-$\mu$ antibody. To visualize better $\mu$s dislocation in vitro and p97 cooperative in a push-pull mechanism in which BiP dissociation from $\mu$s precedes p97 binding.

Nevertheless, our data do not exclude the possibility that BiP and p97 cooperate in a push-pull mechanism in which BiP dissociation from $\mu$s precedes p97 binding.

In Vitro Dislocation of $\mu$s Utilizes ATP and Microsome-bound Cytosolic Components and Reveals Proteasome-independent and -dependent Consecutive Steps—The coprecipitation of the microsome-bound proteasome and p97 with $\mu$s implied that cytosolic components of the ERAD pathway were recruited to the ER cytosolic face, to operate in dislocation of luminal $\mu$s. Therefore, we hypothesized that isolated microsomes would be sufficient to carry out $\mu$s dislocation in vitro. First, when microsomes isolated from $^{[35]}$S-methionine-labeled cells were incubated with cytosol obtained from unlabeled cells, radiolabeled $\mu$s was recovered in the soluble cytosolic fraction in a time- and ATP-dependent manner (Fig. 4A). When we next replaced cytosol with buffer, $\mu$s was still released in the same manner (Fig. 4B, upper and lower panels). BiP, serving as a control for membrane integrity, remained in the microsomes (Fig. 4B, middle panel). Note that the release was not significantly enhanced by the addition of ATP unless the reaction mixture without the added ATP was also treated with apyrase (Fig. 4B, compare lower and upper panels and graphs). This
reflected the endogenous ATP that remained associated with the isolated microsomes. These findings indicated that indeed, microsome-bound cytosolic components were sufficient to support μs dislocation. Moreover, μs was no longer recovered when microsomes were salt washed prior to their incubation in buffer (Fig. 3C, lanes 1–4), suggesting that essential microsome-bound components were removed by salt wash. Nevertheless, when these salt-washed microsomes were incubated with cytosol, the time- and ATP-dependent dislocation of μs was restored (Fig. 4C, lanes 4–7), indicating that freshly added cytosolic components allowed the resumption of μs dislocation. It should be noted that the cytosol added in this experiment was prepared from COS-7 cells, which do not express μs, suggesting that the relevant cytosolic components were not B cell-specific. Taken together, our data demonstrate the involvement of ER-bound cytosolic components in μs dislocation. Such components are recruited to the ER via interactions that endure microsome preparation but are susceptible to high ionic strength. Finally, the similar proportion (~1/3) of μs which was sensitive to either trypsin (Fig. 1, A and E) or salt wash (Fig. 1F) indicated that the salt wash completely removed μs from the cytosolic face of the microsomes, suggesting that the entire dislocation of μs, from within the ER lumen to the cytosol, was reconstituted in vitro.

The in vitro release of μs to the ATP-supplemented buffer could reflect a step subsequent to passage across the ER membrane which could not be observed in vivo because of the rapid degradation of μs by the proteasome immediately upon release. However, proteasome inhibition also did not result in μs accumulation in cytosol, because μs, which crossed the ER membrane, actually remained associated with the cytosolic face of this organelle (Fig. 1). This raised the intriguing possibility that the step that required the proteolytically active proteasome was the actual release to cytosol rather than the passage of μs across ER membrane. Indeed, in the presence of either MG-132 (Fig. 4B, upper panel and graph) or Z-L3 VS (Fig. 4B, lower panel and graph), the release of μs to the buffer ceased within 15 min of in vitro incubation compared with the continuous release of μs in the absence of proteasome inhibitors. Our combined in vivo and in vitro findings indicate that dislocation of ERAD substrates occurs in two consecutive steps: (i) p97/Cdc48p-dependent but proteasome-independent passage across ER membrane and (ii) proteasome-dependent release to cytosol. This explains why in proteasome-inhibited cells μs accumulated at the cytosolic face of the ER rather than being fully released to the cytosol. The data also implicate the membrane-bound active proteasome in the release of μs from the cytosolic face of the ER. In dissecting dislocation into these two consecutive steps, it is still not clear whether p97/Cdc48p plays a role also in the release to the cytosol, but it is evident that it does play a role subsequent to ubiquitination.

**Fig. 5. Schematic model.** The order of events is marked by numbered arrows. Step 5 may represent two consecutive steps, one blocked in CDC48 at 37 °C and the other in cd48–10 at 30 °C. For step 6, the two alternative options represent degradation in association with the ER membrane (6a, 6b).

**DISCUSSION**

In this work, the lumen-to-cytosol dislocation is biochemically dissected for luminal ERAD substrates, and the proposed coupling between initial steps of ERAD is reevaluated. Coupling between ubiquitination and dislocation has been indicated for luminal CPY* (35, 36), soluble truncated ribophorin I (37), and membrane major histocompatibility complex class I heavy chain (38). Multiquitination in particular is required for dislocation of class I heavy chain (14) and CPY* (16). It implicates multiquitin in a ratcheting mechanism or in active pulling out of ERAD substrates across the ER membrane. Likewise, the active proteasome has been postulated to play a role in extraction of several membrane ERAD substrates (10, 39–41). It is noteworthy that the only luminal ERAD substrates for which the proteolytically active proteasome is implicated in dislocation are the μs Ig heavy chain and κ Ig light chain (8, 9). Based on the failure to detect these luminal ERAD substrates in the cytosol upon blocking the proteasome (Refs. 8 and 9 and this work), here we provide evidence for an alternative dislocation mechanism. We demonstrate that despite the proteasome inhibition, the Ig μs heavy chain crosses the ER membrane and accumulates at the cytosolic face of this membrane as multiquitin conjugates.

Studying ERAD using luminal substrates allows us to define their release to the cytosol as a discrete step in ERAD (Fig. 5). Accordingly, dislocation of luminal substrates is dissected into two consecutive steps, which hitherto were not distinguished: (i) passage across the ER membrane and accumulation of substrates at the cytosolic face of this organelle, hence defining the first station in dislocation; (ii) release of these substrates to cytosol, where they are degraded by the proteasome, hence defining the second station. Actually, the release to the cytosol en route to proteasomal degradation has been demonstrated mostly for integral membrane proteins (e.g. major histocompatibility complex class I heavy chain), for which dislocation entails release from the membrane and keeping the substrate in the second station in a soluble state (14, 26, 42). Nonetheless, defining the first station for membrane substrates is ambiguous because such substrates are inherent membrane-spanning proteins and by that resemble luminal soluble substrates al-
Roles of p97/Cdc48p and Proteasome in ERAD

in vitro evidence. First, the reconstituted cytosolic ERAD component, the AAA-ATPase p97/Cdc48p (Refs. 16, 21, 26–28; for review, see Refs. 46 and 47), which participates in a variety of cellular processes and may chaperone protein unfolding or disassembly (48–51), has been shown to coprecipitate with the membrane ERAD substrate 6myc-Hmg2p in yeast and with the luminal ERAD substrate µs in B cells (21), as well as with the luminal yeast HA-CPY*. Here we demonstrate that only the minor ER-bound subpopulation of p97/Cdc48p, and not its vast soluble pool, interacts with µs, analogously to the proteasome. p97/Cdc48p probably plays a role in the actual passage of luminal ERAD substrates across ER membrane, as demonstrated for yeast HA-CPY*, which lingers in the lumen if Cdc48p fails to function. It remains to be established whether the exclusively membrane- and substrate-associated p97/Cdc48p is the subpopulation specialized in ERAD and whether recruitment to membranes provides mechanistic advantages. If so, this phenomenon can be exploited to improve the efficiency of ERAD in handling aberrant proteins in conformational diseases. The increased levels of ER-bound p97 in proteasome-inhibited cells raise the question of how p97 is recruited to membranes. Being a multiubiquitin binding protein, p97 may be recruited by association with multiubiquitinated ERAD substrates. In addition, recruitment may be regulated by p97 phosphorylation-dephosphorylation cycle (52). The possibility that p97 is associated with the ER because of its high affinity to syntaxin 5 is unlikely because only the p97/p47 complex interacts with syntaxin 5 (53, 54), and p47 has been reported to obstruct rather than assist ERAD (26). Moreover, syntaxin 5 and the p97/p47 complex participate in homotypic membrane fusion (53). Although it has been shown that vesicular trafficking, which may involve homotypic fusion, is essential for ERAD of luminal substrates, it is dispensable for ERAD of membrane substrates (20, 55–57). The p97/Cdc48p complex, on the other hand, is clearly essential for ERAD of both luminal and membrane ERAD substrates (16, 21, 26–28). Regardless of the mechanism, the ER-bound p97 resembles the ER-bound proteasome in the ability of both to interact with luminal substrates only subsequent to their emergence from the ER membrane. Then, the once luminal substrates have no topological barrier to interact with cytosolic p97/Cdc48p.

Two possible modes of substrate recognition by p97/Cdc48p can be envisaged. In the first, p97/Cdc48p interacts directly with a polypeptide segment as soon as it emerges from the ER membrane. This possibility may be supported by our finding that multiubiquitination of HA-CPY* is abolished, and this substrate remains entrapped in the ER lumen when Cdc48p fails to function. Moreover, it has been reported that p97 recognizes nonubiquitinated ERAD substrates (65). In the second, the recognition signal is the multiubiquitin moieties attached to the ERAD substrate, as demonstrated here for the luminal ERAD substrate µs and in agreement with the direct interaction of p97 with multiubiquitin (29, 33). Although this interaction is relatively weak (29, 33), it is proposed to be assisted by the Ufd1/Npl4 complex, which by itself can bind multiubiquitin via the Npl4 zinc finger domain (58, 59). Indeed, multiubiquitinated ERAD substrates bind the p97/Cdc48p–Ufd1/Npl4 complex (26). Interestingly, p97 in the context of p97/p47 complex, which functions in homotypic membrane fusion, binds monotheticaly rather than multiubiquitin (58). Based on the complementary results presented here and by Ye and co-workers (65), a dual function for p97/Cdc48p in dislocation is proposed (Fig. 5). p97/Cdc48p functions during the passage across the ER membrane by recognizing nonubiquitinated polypeptide segments, as well as subsequent to extensive multiubiquitination by recognizing multiubiquitin chains, and delivers them to the ER membrane to the proteasome. The physical interaction of p97/Cdc48p with the proteasome on one hand (60, 61) and with multiubiquitinated ERAD substrates on the other hand (Ref. 21 and this work), may assign p97 the responsibility for targeting multiubiquitinated ERAD substrates to the proteasome, as was suggested recently (47). Combined, the significance of these findings is increased further by the fact that they have been obtained for topologically distinct ERAD substrates, membrane and luminal, and in two distinct systems, yeast and mammalian cells.

In light of the two-step dislocation process, it is interesting to reevaluate the role played by p97/Cdc48p in the single dislocation step of membrane major histocompatibility complex class I heavy chain (26). On the other hand, Ufd1 is implicated in the second dislocation step of luminal CPY*, namely the release to the cytosol (16). Regardless of the substrate, recent reports agree that p97/Cdc48p–Ufd1/Npl4 participates in pulling ERAD substrates from the ER, as indicated by the activation of the unfolded protein response when p97/Cdc48p–Ufd1/Npl4 fails to function (16, 21, 26). Here p97/Cdc48p is shown to be directly involved in the passage of luminal substrates across the ER membrane and probably also in the release of these substrates subsequent to their multiubiquitination. Although p97/Cdc48p cannot initiate this passage, this chaperone can interact with luminal substrates as soon as they emerge from the ER membrane. To that effect, it is interesting that analogously to other luminal ERAD substrates (32), BiP appears to initiate µs dislocation. Nonetheless, µs dissociates from luminal BiP before it associates with cytosolic p97 (Fig. 5, step 3).

In our working model (Fig. 5), pleiotropic p97/Cdc48p AAA-
ATPase may coordinate ERAD events at the cytosolic face of the ER. The p97/Cdc48p recruited to the ER membrane can bind emerging luminal substrates either as nonubiquitinated or after multiquitination at least at a single lysine residue (step 3). Exploiting ATP-dependent conformational changes (62), ER-bound p97/Cdc48p may pull the substrates across the ER membrane to allow further multiquitination to proceed (step 4). The last step may be assisted by E4/Ub2, which interacts with p97/Cdc48p (63). Along with this model, multiquitination does not appear to serve as a ratcheting molecule, but rather as a recognition signal for p97 (64). The ER-bound p97 can recruit the proteasome to the cytosolic face of the ER, possibly in an ATP-dependent manner, and/or present the multiquitinated substrates to the ER-bound proteasome (steps 5 and 6a). The proteasome, which executes the ultimate degradation steps in the ERAD pathway, may cooperate with 97/Cdc48p in the release of the substrate to the cytosol (step 6a).

Acknowledgments—We are indebted to K. U. Fröhlich for the cdc48 strains, A. Cooper for the HA-CPY* plasmid, R. Kopito for the /H9262 manuscript. laboratory members for helpful discussions and critical reading of the

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Distinct Steps in Dislocation of Luminal Endoplasmic Reticulum-associated Degradation Substrates: ROLES OF ENDOPLASMIC RETICULUM-BOUND p97/Cdc48p AND PROTEASOME

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J. Biol. Chem. 2004, 279:3980-3989.
doi: 10.1074/jbc.M309938200 originally published online November 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309938200

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