Polyubiquitin Serves as a Recognition Signal, Rather than a Ratcheting Molecule, during Retrotranslocation of Proteins across the Endoplasmic Reticulum Membrane*

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Polyubiquitination is required for retrotranslocation of proteins from the endoplasmic reticulum back into the cytosol, where they are degraded by the proteasome. We have tested whether the release of a polypeptide chain into the cytosol is caused by a ratcheting mechanism in which the attachment of polyubiquitin prevents the chain from moving back into the endoplasmic reticulum. Using a permeabilized cell system in which major histocompatibility complex class I heavy chains are retrotranslocated under the influence of the human cytomegalovirus protein US11, we demonstrate that polyubiquitination alone is insufficient to provide the driving force for retrotranslocation. Substrate release into the cytosol requires an additional ATP-dependent step. Release requires a lysine 48 linkage of ubiquitin chains. It does not occur when polyubiquitination of the substrate is carried out with glutathione S-transferase (GST)-ubiquitin, and this correlates with poly-GST-ubiquitin not being recognized by a ubiquitin-binding domain in the Ufd1-Npl4 cofactor of the ATPase p97. These data suggest that polyubiquitin does not serve as a ratcheting molecule. Rather, it may serve as a recognition signal for the p97-Ufd1-Npl4 complex, a component implicated in the movement of substrate into the cytosol.

In eukaryotic cells, a quality control system in the endoplasmic reticulum (ER)† ensures that only proteins with a native, folded conformation leave the organelle for other destinations, such as the plasma membrane. Misfolded proteins that cannot reach their native state are retrotranslocated from the ER into the cytosol, where they are subsequently degraded by the proteasome (1–3). This cellular pathway is hijacked by certain viruses, such as the human cytomegalovirus. Two human cytomegalovirus proteins, US2 and US11, are able to direct newly synthesized major histocompatibility complex (MHC) class I heavy chains into the ER degradation pathway (4–7). Human MHC class I heavy chain is a 43-kDa type I transmembrane protein with a large luminal/extracellular domain and a short cytosolic tail. The protein is initially inserted into the ER membrane and glycosylated, but under the influence of either US2 or US11, it is rapidly moved into the cytosol, where its N-glycan is cleaved off and the polypeptide chain is degraded by the proteasome (5). The absence of MHC class I molecules at the cell surface allows the virus to propagate without the infected cell being detected by cytotoxic T cells.

Most substrates destined to be degraded are polyubiquitinated while undergoing retrotranslocation. Our previous results showed that polyubiquitination is not only required for degradation by the proteasome but also for retrotranslocation per se (8). Lysine 48-linked polyubiquitin chains are required for protein degradation by the proteasome (9, 10), but given that other linkages are required for other processes (11–15), it would be important to know which kind of polyubiquitin chains are involved in retrotranslocation. The role of polyubiquitination in retrotranslocation also remained unclear. In one model, polyubiquitin provides the driving force for moving the substrate into the cytosol by acting as a ratcheting molecule (for discussion, see Ref. 3). The attachment of the bulky polyubiquitin moiety to a polypeptide segment on the cytosolic side of the membrane would bias random movements of the substrate across the ER membrane by preventing it from sliding back into the ER lumen. This model would be analogous to the one describing posttranslational translocation in the forward direction. In this case, the binding of the luminal chaperone BiP prevents the translocating polypeptide chain from moving back into the cytosol, eventually resulting in its complete transport into the ER lumen (16). If a polyubiquitin-mediated ratcheting mechanism functioned in a similar way in retrotranslocation, polyubiquitination alone should be sufficient to move a substrate into the cytosol. Alternatively, it is possible that the polyubiquitin chain serves as a recognition signal for a downstream component. One candidate for this downstream component is the ATPase p97 (called Cdc48 in yeast), which together with its cofactor Ufd1-Npl4 has been implicated in moving polypeptides from the ER membrane into the cytosol (17–21).

In this study, we have addressed the role of polyubiquitination in retrotranslocation. We show that polyubiquitination is not sufficient to move a substrate into the cytosol. Rather, a subsequent ATP-dependent step is required, during which polyubiquitin in a lysine 48 linkage serves as a recognition signal. Our in vitro experiments suggest that the downstream component may be the p97-Ufd1-Npl4 complex.
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EXPERIMENTAL PROCEDURES

Pulse-Chase Analysis with Permeabilized Cells—Control and US11-expressing U373-MG astrocytoma cells (4) were cultured as described previously (7). The cells were detached from tissue culture flasks with trypsin and incubated in suspension in methionine- and cysteine-free Dulbecco’s minimal essential medium for 1 h at 37 °C. Where indicated, 50 μM of proteasome inhibitor MG-115 (Calbiochem) was present during incubation. The cells resuspended at 1 × 10^7/ml were pulse-labeled for 3–5 min at 37 °C in 290 μCi/ml [35S]methionine and [35S]cysteine (New England Nuclear). They were lysed and centrifuged at 1.6 × 10^6 cells/ml in PB (25 mM Hepes, pH 7.3, 115 mM potassium acetate, 5 mM sodium acetate, 2.5 mM MgCl_2, 0.5 mM EDTA, and 0.04% digitonin (Merck; purified as described in Ref. 22) and protease inhibitor mix (10 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml elastatin, and 1 μg/ml pepstatin). The samples were centrifuged, and the pellets were washed in PB without digitonin and resuspended in cow liver cytosol. Where indicated 1 μM ubiquitin aldehyde, an inhibitor of deubiquitinating enzymes, was added. The samples were then chase-incubated at 37 °C for different time periods. After centrifugation in a microcentrifuge at 14,000 rpm at 4 °C for 10 min, the supernatant of the samples was removed and saved, whereas the pellet fraction was resuspended in PB.

The lysates were made from the samples, and the immunoprecipitations were carried out as described below. Cow liver cytosol was prepared as described previously (8).

Lysate Preparation and Immunoprecipitations—The lysates were made from samples corresponding to 1–2 × 10^6 cells in 0.5% Nonidet P-40 (25 mM ethylene glycol, 0.5% NaCl), 50 mM Tris, pH 8.5, 1% Triton X-100, and 10 mM MgCl_2. The buffer also contained 1 μM phenylmethylsulfonyl fluoride and the previously mentioned protease inhibitor mix. The samples were agitated for 20 min at 4 °C and then clarified by centrifuging in a microcentrifuge at full speed for 10 min. The resulting supernatant was used for immunoprecipitation after SDS and dithiothreitol were added to final concentrations of 0.1% and 0.2%, respectively. Denaturing SDS lysates were made by resuspending cell pellets or cell fractions in 100 μl of 2% SDS and 5 mM dithiothreitol. The samples were heated to 95 °C for 5 min, cooled to room temperature, agitated vigorously, and diluted into Nonidet P-40 buffer so that the final concentrations in the lysate used for immunoprecipitation were ~0.2% SDS, 0.5% dithiothreitol, 0.5% Igepal, 50 mM Tris, pH 8, 150 mM NaCl, and 10 mM MgCl_2. All of the immune complexes were recovered by precipitation with fixed Staphylococcus aureus bacteria (Staph A).

Anti-heavy chain serum and antibodies against bovine ubiquitin were described previously (7). The monoclonal antibody 12C14, which recognizes the influenza hemagglutinin (HA) epitranslocation fragment (3), was used to recover HA protein US11, which was produced in permeabilized U373-MG astrocytoma cells (4). The cells were grown to an the HA expressing U373-MG astrocytoma cells (4) were cultured as described previously (7).

Binding of p97 to Heavy Chains in Permeabilized Cells—The experiments were performed essentially as described (18). The cell fractions were solubilized in 1% Deoxy Big Chap (123N-bis(3-[N-carboxyamido- propyl]aspartyl)cholate), 30 mM Tris/212 pH 7.4, 150 mM potassium acetate, 4 mM magnesium acetate, 1 mM ATP, and protease inhibitors. Immunoprecipitations were carried out with his antibodies followed by a second precipitation with HA antibodies.

Polyubiquitin and Poly-GST-ubiquitin to U61fpl4—Plasmids encoding mammalian C-terminally His-tagged U61, Npl4, and Npl42ZF, lacking the zinc finger domain, were described previously (25). The plasmids pGEX-gp78c and pGEX-MmUBC7 were provided by A. Weissman, p97, GST-gp78c, and GST-Ubc7 were purified as described (18). Polyubiquitin chains were synthesized at 37 °C with 4 μM 5-C, 1 μM GST-gp78c in 25 mM Tris/HCl, pH 7.2, 2 mM magnesium/ATP, 0.1 mM diethiothreitol, 110 mM E1, and 20 μM ubiquitin. The binding experiments were carried out at 4 °C in 0.3 ml of 50 mM Hepes, pH 7.3, 150 mM potassium chloride, 2.5 mM magnesium chloride, 5% glycerol, 2 mM β-mercaptoethanol, 0.1% Triton X-100, 1 mg/ml bovine serum albumin. 20-μl samples of polyubiquitination reactions were incubated with 1 μg of the various purified recombinant proteins. The proteins were then precipitated with specific antibodies, and bound ubiquitin chains were detected by immunoblotting with ubiquitin antibodies.

Retrotranslocation Requires Polyubiquitination and a Subsequent ATP-dependent Step—To study the mechanism of retrotranslocation we used a permeabilized cell system in which native cytotoxic can be replaced with cow liver cytosol (8), allowing for convenient manipulation of cytotoxic. Human astrocytoma cells, stably expressing the human cytomegalovirus protein US11, were pulse-labeled with [35S]methionine and [35S]cysteine. The cells were cultured as described previously (7).

RESULTS

Retrotranslocation Requires Polyubiquitination and a Subsequent ATP-dependent Step—To study the mechanism of retrotranslocation we used a permeabilized cell system in which native cytotoxic can be replaced with cow liver cytosol (8), allowing for convenient manipulation of cytotoxic. Human astrocytoma cells, stably expressing the human cytomegalovirus protein US11, were pulse-labeled with [35S]methionine and [35S]cysteine. The cells were cultured as described previously (7).

When permeabilized cells expressing US11 were incubated with ATP-depleted cow liver cytosol (ΔATP), the degradation of...
the heavy chains was greatly reduced, and the majority of the material stayed on the membrane (lane 16 versus lane 15). As expected from the ATP requirement of the ubiquitin-activating enzyme, no polyubiquitination was observed (lanes 33–36). The addition of AMP-PNP to the ATP-depleted sample did not increase the degradation of heavy chains (lanes 17–20), but it partially restored polyubiquitination (lane 38), in agreement with the expectation that the ubiquitin-activating enzyme can utilize the ATP analog because it hydrolyzes ATP into AMP and PPi (27, 28). The polyubiquitinated chains that were...
formed fractionated with the membrane rather than with the cytosol (lane 40 versus lane 39). The apparent incomplete restoration of polyubiquitination by AMP-PNP may in part be due to deubiquitination occurring during sample preparation (a band at the approximate size of unmodified heavy chains (indicated by an asterisk) was consistently generated even in the presence of protease inhibitors).

To show that this polyubiquitination occurs in a domain of the heavy chain that was originally in the ER lumen, we employed a mutant (K-R) heavy chain, in which the lysines in the C-terminal tail were mutated to arginines. Because ubiquitination can only take place on free amino groups (internal lysine residues or the extreme N terminus), the cytoplasmic domain of this mutant heavy chain cannot be ubiquitinated. For these experiments, we used stable cell lines that express HA-tagged MHC class I heavy chains in addition to US11. In the presence of ATP, a fraction of both the wild-type and the mutant (K-R) heavy chains were deglycosylated during the chase period (Fig. 1B, lane 2 versus lane 1 and lane 10 versus lane 9) and appeared in the cytosolic fraction (lanes 3 and 11). Polyubiquitinated heavy chains accumulated during the chase period (lane 18 versus lane 17 and lane 26 versus lane 25) and were mostly released into the cytosol (lanes 19 and 27). In the presence of AMP-PNP, however, retrotranslocation was blocked (lane 7 versus lane 8 and lane 15 versus lane 16). Polyubiquitinated heavy chains were generated (lanes 22 and 30) but remained in the membrane fraction (lanes 24 and 32).

To detect polyubiquitin chains directly, 125I-ubiquitin was added to cow liver cytosol and incubated with permeabilized astrocytoma cells expressing US11. The samples were subjected to immunoprecipitation with heavy chain antibodies and analyzed by SDS-PAGE and autoradiography (Fig. 2A). Polyubiquitinated heavy chains accumulated over time (lane 2 versus lane 1), and the majority was found in the cytosolic fraction (lane 3 versus lane 4). When ATP was depleted (ΔATP), polyubiquitination was significantly reduced (lanes 5–8). The residual modified chains were found in the membrane rather than in the cytosolic fraction (lane 8 versus lane 7). When AMP-PNP was added to the ATP-depleted sample, polyubiquitination was restored (lane 10), and essentially all modified chains fractionated with the membranes (lane 12 versus lane 11). When similar experiments were performed with US11 cells expressing the HA-tagged wild-type or K-R mutant heavy chains, we observed that in the presence of ATP polyubiquitination occurred on both the wild-type and mutant heavy chains, with most modified chains appearing in the cytosolic fraction (Fig. 1B, lane 4 versus lane 3 and lane 12 versus lane 11). Polyubiquitination was performed with AMP-PNP, the chains failed to be moved into the cytosol and remained associated with the membrane (lane 8 versus lane 12 and lane 16 versus lane 15). These chains appear to have a higher molecular mass than in the presence of ATP and ATP-regenerating system (lanes 5–8 versus lanes 1–4 and lanes 13–16 versus lanes 9–12), which may reflect the existence of an ATP-dependent deubiquitinating step. Taken together, these results indicate that polyubiquitination occurs on a segment that was originally in the ER lumen and

![Fig. 2. ATP is required for both polyubiquitination and a downstream step. A, US11-expressing cells were permeabilized and incubated in cow liver cytosol containing ATP and an ATP-regenerating system (ARS), lacking ATP (ΔATP), or lacking ATP and containing instead AMP-PNP (ΔATP + AMPPNP). 125I-Ubiquitin was added for 45 min at 37 °C, and MHC class I heavy chains were recovered by immunoprecipitation (IP) with αHC. The samples were analyzed by SDS-PAGE and autoradiography. B, as in A, but with US11 cells also expressing HA-tagged wild-type or K-R heavy chains. Immunoprecipitation was carried out with αHA.](#)
that modification of these residues alone is insufficient to move the substrate into the cytosol. An additional ATP-dependent step that requires hydrolysis of the γ-phosphate of ATP appears to be involved.

To provide further evidence that an additional ATP-dependent step is required for the release of heavy chains into the cytosol, we performed experiments in the absence of AMP-PNP, thus excluding the possibility that the ATP analog blocked a step following polyubiquitination. Permeabilized cells labeled with [35S]methionine and [35S]cysteine were resuspended in cow liver cytosol in the absence of ATP and chase-incubated for 20 min. Then either buffer or 0.1 mM ATP was added, and the incubation was continued for another 10 min (30 min total). The polyubiquitinated heavy chains formed during the labeling period stayed on the membrane in the absence of ATP (Fig. 3, lanes 1–7, P). However, when ATP was added, about half of the polyubiquitinated chains were released into the cytosol (lane 13 versus lane 14). In the presence of ATP, some shorter polyubiquitinated species appeared in the membrane fraction, either because existing chains were deubiquitinated or because new polyubiquitin chains were formed. These results confirm that polyubiquitination alone is insufficient and that ATP is required for the release of modified substrate from the ER membrane into the cytosol.

**Lysine 48 Linkage of Polyubiquitin Chains Is Required for Retrotranslocation**—Different lysines in the ubiquitin molecule can serve for chain elongation, resulting in polyubiquitin chains with distinct roles in the cell. For example, polyubiquitin chains with lysine 48 linkages are required for proteasomal degradation (9), whereas chains with lysine 63 linkages are implicated in nonproteolytic events (11, 13–15). We therefore wished to determine whether a specific linkage is required for retrotranslocation but on its own is insufficient, we tested whether polyubiquitin chains may function as a recognition signal. Specifically, we investigated whether modification of polyubiquitin chains by the addition of a GST moiety to the N-terminal part of ubiquitin would influence retrotranslocation. We reasoned that GST-ubiquitin molecules may still be linked with one another to form poly-GST-ubiquitinated heavy chains but that these chains may no longer be recognized by a downstream component.

We incubated [35S]-labeled permeabilized cells with cow liver cytosol supplemented with GST-ubiquitin. Compared with control cow liver cytosol, the appearance of deglycosylated heavy chains in the cytosol was reduced (Fig. 5A, lanes 16–20 versus lanes 11–15). In addition, a high molecular mass species appeared during the chase period, which was not seen in the absence of GST-ubiquitin. The high molecular mass species were GST-ubiquitinated heavy chains as demonstrated by re-immunoprecipitation with either ubiquitin antibodies (bottom panel, lanes 41–45) or GST antibodies (Fig. 5B, lanes 13–16). In most experiments, a fraction of 15–20% of the total population of heavy chains present at the beginning of the chase period were poly-GST-ubiquitinated, but occasionally up to 55% was modified. This is a much higher percentage of modification than seen with wild-type ubiquitin (see lanes 11–15 and 36–40), which may suggest that deubiquitination is impaired by GST-ubiquitin. A significant fraction of GST-ubiquitinated heavy chains were membrane-associated (lane 20 versus lane 19 and lane 25 versus lane 24). Some deglycosylated chains were released into the cytosol even in the presence of GST-ubiquitin (lane 19), and some poly-GST-ubiquitinated molecules could be immunoprecipitated from the cytosolic fraction.
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Polyubiquitin as Recognition Signal during Retrotranslocation (lane 44). This is probably due to chains containing endogenous ubiquitin in addition to GST-ubiquitin. Indeed, when ubiquitin was depleted from the cytosol (8) before the addition of GST-ubiquitin, the appearance of the deglycosylated species was completely blocked, and essentially all modified chains remained in the membrane (lanes 21–25). The maximum amount of GST-ubiquitinated heavy chains was reached after 20–30 min of incubation (Fig. 5B), and these adducts were stable for at least 2 h. Similar results were obtained with both the wild-type and K-R mutant heavy chains (data not shown). The GST-ubiquitin modified heavy chains were sensitive to protease treatment (data not shown), as expected from the fact that ubiquitination is a cytosolic event. These results show that the attachment of multiple GST-ubiquitin molecules to heavy chains is not sufficient for their release into the cytosol. They suggest that poly-GST-ubiquitin prevents retrotranslocation by disrupting a recognition signal for a downstream component. Apparently, the GST moiety also prevents recognition by the deubiquitinating enzymes.

Poly-GST-Ubiquitin Is Not Recognized by a Ubiquitin-binding Domain in the Ufd1-Npl4 Cofactor of the ATPase p97—One of the candidates for a downstream component is the ATPase p97, which has been shown to function together with its cofactor Ufd1-Npl4 in retrotranslocation (17–21). We first tested whether poly-GST-ubiquitination of MHC class I heavy chains would abrogate substrate recognition by p97, which would provide an explanation why these chains can no longer be moved into the cytosol. To determine substrate binding by p97, astrocytoma cells expressing US11 and HA-tagged wild-type heavy chains were treated with proteasome inhibitor, labeled, and permeabilized. The endogenous cytosol was replaced with cow liver cytosol that was either depleted of ubiquitin as described (8) or first depleted and then replenished with ubiquitin or GST-ubiquitin (Fig. 6A). Where indicated, His-tagged recombinant p97 protein was added, and the samples were chased incubated for 30 min and then separated into pellet and supernatant fractions. Immunoprecipitation of the heavy chains bound to U/N and Npl4 was carried out with αHC antibodies (lanes 7 and 8 versus lanes 3 and 4). In the absence of ubiquitin, retrotranslocation was blocked (lanes 9–12). In the presence of ubiquitin, the deglycosylated species appeared in the supernatant, regardless of whether p97 was added or not (upper panel, lanes 4 and 8). In the absence of ubiquitin, retrotranslocation was blocked (lanes 9–12). In the presence of GST-ubiquitin, retrotranslocation was also reduced, and the characteristic high molecular adducts appeared in the membrane fraction (lanes 13–16). To detect p97-associated heavy chains, the samples were first immunoprecipitated with His antibodies and then with HA antibodies (lanes 17–32). Binding of heavy chains was seen regardless of whether or not ubiquitin was present during the incubation (lanes 21–24 versus lanes 25–28), demonstrating that p97 can interact with nonmodified substrate. In the absence of His-p97, no material was immunoprecipitated (lanes 17–20). p97 also interacted with poly-GST-ubiquitinated heavy chains (lanes 29–32). Thus, the fact that poly-GST-ubiquitinated heavy chains cannot be released into the cytosol cannot be explained by disruption of substrate recognition by p97.

Because previous experiments implicated the cofactor U61-Npl4 in the recognition of the polyubiquitin chains, we next tested whether poly-GST-ubiquitin may not be recognized by this cofactor, providing a possible explanation for why this modification disrupts retrotranslocation. Polyubiquitin chains were synthesized in vitro using purified recombinant proteins. The assay contained ubiquitin, the E1, the E2, and a GST fusion to a cytosolic fragment of the E3. We employed Ubc7 and gsp78 because they are known to be involved in the ER degradation of at least some proteins (29–32). When all of the components were incubated together in the presence of ATP, polyubiquitin chains were synthesized (Fig. 6B, upper panel, lane 1). These chains were tested for interaction with the Ufd1-Npl4 (UN) complex. The mammalian cofactor complex has two polyubiquitin-binding domains: one in Ufd1 and one in a zinc finger domain of Npl4. Because the Npl4 ubiquitin-binding domain is lacking in yeast (33) and the complex likely functions analogously to the mammalian one, the binding site in Ufd1 appears to be most important in retrotranslocation. We therefore tested the binding of both wild-type Ufd1-Npl4 and of a mutant complex that lacked the zinc finger domain in Npl4 (UNΔZF). Polyubiquitin chains were incubated with these complexes, and binding was assessed by immunoprecipitation with Ufd1 antibodies followed by immunoblotting with ubiquitin antibodies. With the wild-type complex, a significant fraction of the polyubiquitin chains was bound (Fig. 6B, upper panel, lane 3; the lower panel demonstrates coprecipitation of Ufd1 and Npl4). As expected, the mutant complex (UNAZF), containing only the Ufd1 binding site for polyubiquitin, had reduced but still significant binding activity (lane 4). Next, we performed binding experiments with poly-GST-ubiquitin instead of wild-type polyubiquitin. The polymerization reaction with GST-ubiquitin was less efficient, but high molecular mass adducts were generated (lane 5). The poly-GST-ubiquitin chains bound to UN but not to UNAZF as demonstrated with both ubiquitin and GST blots (lanes 7 and 8 versus lanes 3 and 4 in upper and lower panels). The generated poly-GST-ubiquitin chains were linked through lysine 48 of ubiquitin, as demonstrated by the fact that poly-GST-ubiquitin chains could not generated with GST-K48R or GST-K0 ubiquitin (which lacks all 7 lysines in ubiquitin) (Fig. 6C, compare lane 4 with lanes 5 and 6). Taken together, these results show that poly-GST-ubiquitin chains are not recognized by the Ufd1 ubiquitin-binding domain of the cofactor Ufd1-Npl4, even though the chains have the correct ubiquitin linkage. On the other hand, intact Ufd1-Npl4 can interact with poly-GST-ubiquitin, likely through the zinc finger domain in Npl4. Our data are consistent with the hypothesis that poly-GST-ubiquitinated heavy chains may not be recognized by the cofactors of p97 during retrotranslocation.

**DISCUSSION**

Our previous results showed that polyubiquitination is required for retrotranslocation of MHC class I heavy chains from the ER membrane into the cytosol (8). Now, we demonstrate...
that polyubiquitination is insufficient for retrotranslocation. When the modification reaction was performed in the presence of AMP-PNP or when GST-ubiquitin instead of wild-type ubiquitin was employed in the reaction, polyubiquitinated heavy chains stayed on the membrane and were not moved into the cytosol. In addition, membrane-associated heavy chains modified with wild-type polyubiquitin could be released into the cytosol by the addition of ATP. Together, these data show that there is an ATP requirement in at least two steps of retrotranslocation: one for polyubiquitination, which can be satisfied by AMP-PNP, and the other for a downstream component that requires hydrolysis of the $\gamma$-phosphate of ATP. Because polyubiquitination alone was insufficient to move a polypeptide into the cytosol, we conclude that the driving force for retrotranslocation is not provided by a simple ratcheting mechanism. This is in contrast to posttranslational translocation in the forward direction, in which a secretory protein can be moved completely into the ER lumen by a ratcheting mechanism (16). Although polyubiquitination does not suffice to move a polypeptide chain completely into the cytosol, it may still prevent movements back into the ER lumen. The initial steps of retrotranslocation must be independent of polyubiquitina-

**Fig. 5.** GST-ubiquitin stabilizes MHC class I heavy chains in the membrane. US11 cells and control cells were incubated with proteasome inhibitors and $[^{35}S]$methionine/$[^{35}S]$cysteine, permeabilized, and incubated in cow liver cytosol or cow liver cytosol first depleted of ubiquitin ($\Delta$Ub) in the presence or absence of GST-ubiquitin (GST-Ub). After incubation at 37 °C for different time periods, the lysates were analyzed either directly or after fractionation into supernatant ($S$) and membrane pellet ($P$). The heavy chains were recovered by immunoprecipitation ($IP$) with heavy chain antibodies ($\alpha$HC, lanes 1–25, and $\alpha$Ub, lanes 1–8), followed by a second round of immunoprecipitation with ubiquitin antibodies ($\alpha$Ub, lanes 26–45, and $\alpha$GST, lanes 9–12) or GST antibodies (B, lanes 13–16). $HC$ – $CHO$ and $HC$ + $CHO$ indicate heavy chains without or with carbohydrate chain, respectively. Molecular mass marker ($M$) bands are 220, 97, 66, 45, and 30 kDa. The band migrating around 43 kDa is possibly heavy chain deubiquitinated during sample preparation (*).
tion of the substrate, because the modification occurs on segments that were originally in the ER lumen. It may not be too surprising that polyubiquitination does not provide a simple ratcheting mechanism, because the distances between consecutive lysine residues, the usual attachment sites in a polypeptide chain, would make the ratchet in general inefficient. In addition, some proteins contain few or even no lysines.

Our data not only indicate the existence of an ATP-dependent step following polyubiquitination but also suggest that polyubiquitin serves as a recognition signal for a downstream component, FIG. 6.

**A** Interaction of the ATPase p97 and its cofactor Ufd1-Npl4 with poly-GST-ubiquitin. A, US11 cells stably expressing HA-tagged wild-type heavy chain were labeled with \[^{35}S\]methionine/\[^{35}S\]cysteine and permeabilized. The cell pellet was resuspended in cow liver cytosol that was either depleted of ubiquitin (\(\Delta\text{Ub}\)) or replenished with ubiquitin or GST-ubiquitin. Where indicated recombinant His-tagged p97 was added. The samples were then incubated at 37 °C for 0 or 30 min and separated into supernatant (S) and membrane pellet (P) fractions. One portion was subjected directly to immunoprecipitation (IP) with aHA (lanes 1–16), and another was first immunoprecipitated with His antibodies for p97, followed by a second round of immunoprecipitation with aHA (lanes 17–32). All of the samples were analyzed by SDS-PAGE and autoradiography. After immunoprecipitation with His antibodies, part of the sample was analyzed by Coomassie Blue staining to show amounts of precipitated His-tagged p97 (lanes 33–48). B, polyubiquitin chains were synthesized in vitro using ubiquitin or GST-ubiquitin as substrate (see “Experimental Procedures”). The samples were incubated with buffer (–) or with the complex of purified Ufd1 and either Npl4 (U/N) or Npl4 lacking the zinc finger domain (U/NZF). Bound polyubiquitin chains were analyzed after immunoprecipitation with Ufd1 antibodies by blotting with ubiquitin antibodies. The **lower panel** shows an immunoblot with antibodies to Ufd1, Npl4, and GST. 30% of the material used for the binding experiments was subjected directly to SDS-PAGE (input). C, polyubiquitin chains were synthesized in vitro using either wild-type ubiquitin, a GST fusion to wild-type ubiquitin, or GST-fusions to K48R or K0 ubiquitin (in the latter all lysines are changed to arginines). In lane 3, the reaction contained wild-type ubiquitin and GST. The chains were separated by SDSPAGE and analyzed by immunoblotting with ubiquitin antibodies.
because a substrate that is poly-GST-ubiquitinated could not be moved into the cytosol. This idea of polyubiquitin as a recognition signal is strengthened by the fact that a specific lysine 48 linkage within the polyubiquitin chain is required for retrotranslocation, the same linkage that is required for the proteasome that acts subsequent to retrotranslocation in substrate degradation.

The simplest model is that a downstream component recognizes polyubiquitin and functions in an ATP-dependent process. Previously, the ATPase p97 and its cofactor Ufd1-Npl4 have been implicated in retrotranslocation. Our data suggest that this complex may be involved in the ATP- and polyubiquitin-dependent step. A requirement for ATP hydrolysis by p97 would explain why AMP-PNP did not allow retrotranslocation. Furthermore, a p97 mutant defective in ATP hydrolysis leads to a similar phenotype as AMP-PNP, *i.e.*, the accumulation of polyubiquitinated substrate on the ER membrane. The binding of polyubiquitin to the p97 complex is also consistent with it being the immediate downstream component. The binding occurs to an N-terminal domain of Ufd1 in the cofactor complex. Although the zinc finger domain in Npl4 can also bind polyubiquitin (25), this interaction is likely not important for retrotranslocation. It does not discriminate between lysine 48 and lysine 63 linkages, and the yeast homolog of Npl4 lacks the zinc finger domain (33) altogether but likely functions similarly to the mammalian protein in retrotranslocation. The fact that this domain can even interact with mono-ubiquitin (34) may explain the interaction we observe with polyubiquitin. The fact that this domain can even interact with mono-ubiquitin and functions in an ATP-dependent process may be similar to the role suggested for deubiquitinating enzymes in the proteasome or deubiquitinating enzymes, are also prevented from binding and cause the retrotranslocation defect. For example, deubiquitinating enzymes may be important to remove the polyubiquitin chains from polypeptides so that they can be moved through the relatively narrow pore in the double-barrel structure of the p97 ATPase (35). This function would be similar to the role suggested for deubiquitinating enzymes in the degradation of proteins by the proteasome. The inhibitory effect of AMP-PNP on deubiquitination seen in our experiments could be explained by an enzyme similar to that found on the proteasome (26, 36).

Interestingly, p97 itself can bind to both nonubiquitinated and polyubiquitinated substrate molecules. This suggests that there are two consecutive interactions of the p97-Ufd1-Npl4 complex with retrotranslocating heavy chains. It first binds to nonubiquitinated segments of the substrate and then, following ubiquitination, to the polyubiquitin chain. How exactly the polypeptide substrate would subsequently be moved into the cytosol remains unclear, and our data also do not exclude that a component other than the p97-Ufd1-Npl4 complex could be involved. However, one of the possibilities is that the interaction of the cofactor with the polyubiquitin chain activates the ATPase p97 to "pull" the polypeptide chain out of the membrane.

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