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

Dennis Flierman†, Yihong Ye‡, Min Dai, Vincent Chau§, and Tom A. Rapoport¶‡‡

From the †Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, the ¶Howard Hughes Medical Institute and Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, and the §Department of Cellular and Molecular Physiology, Penn State University College of Medicine, Hershey, Pennsylvania 17033

Polyubiquitin 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 mediated 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 (1–5). 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 (7–9). 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.
EXPERIMENTAL PROCEDURES

Pulse-Chase Analysis with Permeabilized Cells—Control and US11-expressing U373-MG astrocytoma cells (4) were cultured as described previously (5). The cells were detached from tissue culture flasks by 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 250 μCi/ml [35S]-methionine and [35S]cysteine (17) in 25 mM Tris/HCl, pH 7.4, 150 mM sodium 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.

Binding of Polyubiquitin and Poly-GST-ubiquitin to U61-fcp—Plasmids encoding mammalian C-terminally His-tagged U61, Np44, and Np44fcp, lacking the zinc finger domain, were described previously (25). The plasmids pGEX-gp78c and pGEX-MnmU8C7 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 Ubc7, 1 μM GST-gp78c and 2 μM GST-Ubc7 in 25 mM Tris/HCl, pH 7.2, 2 mM magnesium/ATP, 0.1 mM dithiothreitol, 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 phosphate, 2.5 mM magnesium chloride, 5% 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.

Miscellaneous Methods—For ATP depletion, the cytosol was incubated for 20 min at 37 °C with 0.1 unit/μl hexokinase and 20 mM glucose where indicated. ATP was measured during the ATP-regenerating system (24). The reaction was allowed to proceed for 10 min at 37 °C. The reaction mixture was subsequently transferred to glutathione-Sepharose 4B beads and incubated at 4 °C for 15–30 min. The non-bound material was frozen at −80 °C.

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 cytosol can be replaced with cow liver cytosol (8), allowing for convenient manipulation of cytosol. Human astrocytoma cells, stably expressing the human cytomegalovirus protein US11, were pulse-labeled with [35S]methionine and [35S]cysteine. The cells were permeabilized with addition of a low concentration of digitonin, pelleted, and resuspended in cytosol from cow liver in the presence of ATP and an ATP-regenerating system. After chase incubation at 37 °C, one portion of the samples was analyzed directly, and the other was separated into membrane pellet and supernatant fractions. The samples were solubilized in detergent, subjected to immunoprecipitation with antibodies to MHC class I heavy chains (α2C), and analyzed by SDS-PAGE and autoradiography (Fig. 1A, lanes 9–12). As reported previously (5, 7), the heavy chains were degraded during the chase period (lane 10 versus lane 9). In addition, the characteristic deglycosylated, faster migrating heavy chain species, which fractionated with the cytosol, appeared (lane 11 versus lane 12). To detect polyubiquitinated heavy chains, a portion of the samples was subjected to a second round of immunoprecipitation with ubiquitin antibodies (lanes 29–32). Polyubiquitinated heavy chains, appearing as a smear of high molecular mass species, accumulated over time (lane 30 versus lane 29). Most of the polyubiquitinated heavy chains were found in the cytosolic fraction (lane 31 versus lane 32), indicating that they had been released from the ER membrane. In control cells not expressing US11, the heavy chains were stable (lanes 1–8), and no polyubiquitination was observed (lanes 21–28). It should be noted that previous experiments showed that polyubiquitination is required for retrotranslocation (8), implying that the majority of the retrotranslocated heavy chains, visible in the cytosolic fraction as the deglycosylated species, must have undergone deubiquitination, even in the presence of 1 μM ubiquitin-aldehyde.

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 PP$_i$ (27, 28). The polyubiquitinated chains that were

Fig. 1. Polyubiquitinated heavy chains can be formed and stabilized on the membrane in the presence of AMP-PNP. A, control cells or cells expressing the human cytomegalovirus protein US11 were labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine, permeabilized, and incubated in cow liver cytosol either in the presence of ATP and an ATP-regenerating system (ARS), in the absence of ATP (ΔATP), or in the absence of ATP but the presence of AMP-PNP (ΔATP + AMP-PNP). After incubation for 0 or 45 min at 37 °C, one portion of the sample was directly analyzed, the other was fractionated into supernatant (S) and membrane pellet (P). The samples were subjected to immunoprecipitation (IP) with αHC and to a second round of immunoprecipitation with αUb. Analysis was done by SDS-PAGE and autoradiography. The band migrating around 43 kDa is possibly heavy chain, deubiquitinated during sample preparation (*). HC – CHO and HC + CHO indicate heavy chains without or with carbohydrate chain, respectively. 1 μM ubiquitin-aldehyde was present during incubation. B, US11 cells stably expressing either HA-tagged wild-type or K-R heavy chains were treated as in A, except that cells were preincubated with proteasome inhibitor and that recovery of the heavy chains from the lysates was carried out with αHA. 1 μM ubiquitin-aldehyde was present during incubation.
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). When 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 7 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...
that modification of these residues alone is insufficient to move the substrate into the cytosol. An additional ATP-depen-
dent 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 incuba-
tion was continued for another 10 min (30 min total). The polyubiq-
uitinated 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 polyubiqui-
nated 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 exist-
ing chains were deubiquitinated or because new polyubiquitin
chains were formed. These results confirm that polyubiquitina-
tion 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 there-
fore wished to determine whether a specific linkage is required for retrotranslocation. US11 cells were pulse-labeled with
[35S]methionine/[35S]cysteine, permeabilized, and resuspended in ubiquitin-depleted cytosol replenished with buffer, wild-type
ubiquitin, or ubiquitin mutants lacking specific attachment
sites (K48R or K63R ubiquitin). The cells were incubated for 30
min at 37 °C, and then a portion of the samples was analyzed
directly, whereas the other was separated into a membrane
pellet and a cytosolic supernatant fraction. After solubilization
in detergent, the samples were subjected to immunoprecipita-
tion with αHC and analyzed by SDS-PAGE and autoradiogra-
phy. In the absence of ubiquitin (Fig. 4, lanes 1–4), MHC class
I heavy chains were retained in the membrane (lane 4). In the
presence of wild-type ubiquitin (lanes 5–8), the deglycosylated
heavy chain species appeared in the cytosolic fraction (lane 7
versus lane 8). When the K48R mutant was present (lanes
9–12), however, hardly any deglycosylated species was ob-
erved in the cytosolic fraction (lane 11). The K63R mutant
behaved like wild-type ubiquitin (lanes 13–16 versus lane
5–8). These results indicate that polyubiquitin chains with lysine 48
linkages are required for retrotranslocation.

Poly-GST-Ubiquitination of MHC Class I Heavy Chain
Prevents Its Retrotranslocation—Because polyubiquitination with a
specific linkage is required for retrotranslocation but on its own is
insufficient, we tested whether polyubiquitin chains may func-
tion 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 ret-
rotranslocation. 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 con-
trol 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 ap-
ppeared 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 mole-
cules could be immunoprecipitated from the cytosolic fraction.
Polyubiquitin as Recognition Signal during Retrotranslocation

**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-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, K48R ubiquitin, or K63R ubiquitin for 30 min. The samples were analyzed either directly or after fractionation into supernatant (S) and membrane pellet (P) fractions. Immunoprecipitation (IP) was carried out with αHC. The samples were analyzed by SDS-PAGE and autoradiography. HC−CHO and HC + CHO indicate heavy chains without or with carbohydrate chain, respectively.

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, K48R ubiquitin, or K63R ubiquitin for 30 min. The samples were analyzed either directly or after fractionation into supernatant (S) and membrane pellet (P) fractions. Immunoprecipitation (IP) was carried out with αHC. The samples were analyzed by SDS-PAGE and autoradiography. HC−CHO and HC + CHO indicate heavy chains without or with carbohydrate chain, respectively.

**FIG. 4. Retrotranslocation requires a lysine 48 linkage in polyubiquitin chains.** US11-expressing cells were pulse-labeled with [35S]methionine/[35S]cysteine, permeabilized, incubated in cow liver cytosol depleted of ubiquitin (ΔUb), and supplemented with buffer, wild-type (wt) ubiquitin, K48R ubiquitin, or K63R ubiquitin for 30 min. The samples were analyzed either directly or after fractionation into supernatant (S) and membrane pellet (P) fractions. Immunoprecipitation (IP) was carried out with αHC. The samples were analyzed by SDS-PAGE and autoradiography. HC−CHO and HC + CHO indicate heavy chains without or with carbohydrate chain, respectively.
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 γ-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).

**Fig. 5. GST-ubiquitin stabilizes MHC class I heavy chains in the membrane.** US11 cells and control cells were incubated with proteasome inhibitors and [35S]methionine/[35S]cysteine, permeabilized, and incubated in cow liver cytosol or cow liver cytosol first depleted of ubiquitin (ΔUb) 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 (A, lanes 1–25, and B, lanes 1–8), followed by a second round of immunoprecipitation with ubiquitin antibodies (A, lanes 26–45, and B, 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. 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 [35S]methionine/[35S]cysteine and permeabilized. The cell pellet was resuspended in cow liver cytosol that was either depleted of ubiquitin (ΔUb) 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 αHA (lanes 1–16), and another was first immunoprecipitated with His antibodies for p97, followed by a second round of immunoprecipitation with αHA (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 SDS-PAGE 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 the same phenotype as AMP-PNP, i.e., the accumulation of polyubiquitinated substrate on the ER membrane. 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 analogously 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 poly-GST-ubiquitin. In contrast, the ubiquitin-binding domain in Ufd1 is specific for lysine 48 linkages in poly ubiquitin chains and does not recognize lysine 63 linkages, consistent with our finding that lysine 48 is required for retrotranslocation. We have found that Ufd1-Npl4 lacking the zinc finger domain in Npl4 does not bind poly-GST-ubiquitin, even though the chains have the correct lysine 48 linkages. The lack of interaction of poly-GST-ubiquitin with the ubiquitin-binding domain in Ufd1 could explain why this modification blocks the movement of MHC class I heavy chains into the cytosol. However, it is possible that other polyubiquitin-binding proteins, such as 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.

Acknowledgments—We thank Thomas Sommer for providing the GST-ubiquitin and GST-K48R constructs and Gia Voeltz, Jing Ye, and Ami Navon for comments on the manuscript. We thank Anna Borodovsky and her supervisor Hilde Ploegh for help with the iodination of ubiquitin.

REFERENCES

1. Brodsky, J. L., and McCracken, A. A. (1999) Semin. Cell Dev. Biol. 10, 507–513
2. Ellgaard, L., and Helenius, A. (2001) Curr. Opin. Cell Biol. 13, 431–437
3. Tsai, B., Ye, Y., and Rapoport, T. A. (2002) Nat. Rev. Mol. Cell. Biol. 3, 246–255
4. Jones, T. R., Hannon, L. K., Sun, L., Slater, J. S., Stenberg, E. M., and Campbell, A. E. (1995) J. Virol. 69, 4830–4841
5. Wiertz, E. J., Jones, T. R., Sun, L., Bogoy, M., Geuze, H. J., and Ploegh, H. L. (1996) Cell 84, 769–779
6. Wiertz, E. J., Tortorella, D., Bogoy, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996) Nature 384, 432–438
7. Shou, C. E., Story, C. M., Rapoport, T. A., and Ploegh, H. L. (1999) J. Cell Biol. 147, 45–58
8. Shamu, C. E., Flierman, D., Ploegh, H. L., Rapoport, T. A., and Chau, V. (2001) Mol. Biol. Cell 12, 2546–2555
9. Chau, V., Tobias, J. W., Bachmair, A., Marrriott, D. E., Jackson, N., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583
10. Gregori, L., Foncillas, J., Goujon, G., and Chau, V. (1999) J. Biol. Chem. 274, 8354–8357
11. Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995) Mol. Cell. Biol. 15, 1265–1273
12. Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17456
13. Hofmann, R. M., and Pickart, C. M. (1999) Cell 96, 645–653
14. Spence, J., Gali, R. R., Dittmar, G., Sherman, P., Karin, M., and Finley, D. (2000) Cell 102, 67–76
15. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Cell 103, 351–361
16. Matlack, K. E., Misselwitz, B., Plath, K., and Rapoport, T. A. (1999) Cell 97, 553–564
17. Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001) Mol. Biol. Cell 12, 4114–4129
18. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) Nature 414, 652–656
19. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) EMBO J. 21, 615–621
20. Jarosch, E., Taxis, V., Volkwein, C., Bordalio, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Nat. Cell Biol. 4, 134–139
21. Rabievich, E., Keren, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (2002) Mol. Cell. Biol. 22, 626–634
22. Gerlich, D., and Rapoport, T. A. (1995) Cell 75, 615–630
23. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 421–466, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Feldman, R. M., Correll, C. C., Kaplan, B. L., and Deshaies, R. J. (1997) Cell 91, 221–230
25. Meyer, H. H., Wang, Y., and Warren, G. (2002) EMBO J. 21, 5465–5462
26. Eyton, E., Armon, T., Heller, H., Beck, S., and Herschkowitz, A. (1993) J. Biol. Chem. 268, 4668–4674
27. Johnston, N. L., and Cohen, R. E. (1991) Biochemistry 30, 7514–7522
28. Pickart, C. M., Kasperek, E. M., Beal, R., and Kim, A. (1994) J. Biol. Chem. 269, 7115–7123
29. Biederer, T., Volkwein, C., and Sommer, T. (1996) EMBO J. 15, 2069–2076
30. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) Science 273, 1725–1728
31. Hampton, R. Y., and Shrikant, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12944–12948
32. Fang, S., Ferriere, M., Yang, C., Jensen, J. P., Tiwari, S., and Weissman, A. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14422–14427
33. Hitchcock, A. L., Krebber, H., Fretat, S., Lin, A., Latterich, M., and Silver, P. A. (2001) Mol. Biol. Cell 12, 3226–3241
34. Wang, B., Alam, S. L., Meyer, H. H., Payne, M., Stemmler, T. L., Davis, D. R., and Jentsch, S. (2002) Mol. Biol. Cell 12, 615–621
35. Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlow, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., and Freemont, P. S. (2000) Mol. Cell 6, 1473–1484
36. Verma, R., Arvind, L., Ozawa, R., McDonald, W. H., Yates, J. R. III, Koonin, E. V., and Deshaies, R. J. (2002) Science 296, 611–615