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E2-25K mediates US11-triggered retro-translocation of MHC class I heavy chains in a permeabilized cell system

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In cells expressing human cytomegalovirus US11 protein, newly synthesized MHC class I heavy chains (HCs) are rapidly dislocated from the endoplasmic reticulum (ER) and degraded in the cytosol, a process that is similar to ER-associated degradation (ERAD), the pathway used for degradation of misfolded ER proteins. US11-triggered movement of HCs into the cytosol requires polyubiquitination, but it is unknown which ubiquitin-conjugating and ubiquitin-ligase enzymes are involved. To identify the ubiquitin-conjugating enzyme (E2) required for dislocation, we used a permeabilized cell system, in which endogenous cytosol can be replaced by cow liver cytosol. By fractionating the cytosol, we show that E2-25K can serve as the sole E2 required for dislocation of HCs *in vitro*. Purified recombinant E2-25K, together with components that convert this E2 to the active E2-ubiquitin thiolester form, can substitute for crude cytosol. E2-25K cannot be replaced by the conjugating enzymes HsUbc7/Ube2G2 or Ube2G1, even though HsUbc7/Ube2G2 and its yeast homolog Ubc7p are known to participate in ERAD. The activity of E2-25K, as measured by ubiquitin dimer formation, is strikingly enhanced when added to permeabilized cells, likely by membrane-bound ubiquitin protein ligases. To identify these ligases, we tested RING domains of various ligases for their activation of E2-25K *in vitro*. We found that RING domains of gp78/AMFR, a ligase previously implicated in ERAD, and MARCHVII/axotrophin, a ligase of unknown function, greatly enhanced the activity of E2-25K. We conclude that in permeabilized, US11-expressing cells polyubiquitination of the HC substrate can be catalyzed by E2-25K, perhaps in cooperation with the ligase MARCHVII/axotrophin.

cytomegalovirus | ubiquitin-mediated proteolysis | ubiquitin-conjugating enzyme | ubiquitin protein ligase

The expression level of a large set of proteins in eukaryotes is regulated by proteolysis, in which proteins are modified with polyubiquitin chains and subsequently degraded by the 26S proteasome (1, 2). Ubiquitin-mediated proteolysis also functions in protein quality control, resulting in the degradation of misfolded or damaged proteins. A particularly well studied quality-control system is found in the endoplasmic reticulum (ER) (3, 4). A large number of diseases are known in which mutant proteins fail to fold properly in the ER and are degraded. Examples include the CFTR protein in cystic fibrosis, α1-antitrypsin in childhood liver disease and adult emphysema, low-density lipoprotein receptor in familial hypercholesterolemia and myeloperoxidase deficiency, and insulin receptor in type A insulin resistance (1, 5). It was initially believed that protein degradation occurs inside the ER (6), but it is now accepted that misfolded proteins are transported back into the cytosol, a process termed dislocation or retro-translocation, before they are degraded by the proteasome (4, 7). In this ER-associated degradation (ERAD) pathway most substrates are polyubiquitinated before being moved into the cytosol (8, 9).

Polyubiquitination of proteins requires the concerted action of a ubiquitin-conjugating enzyme (E2) and a ubiquitin protein ligase (E3). Ubiquitin is linked via its C-terminal carboxyl group to a cysteine in an E2 to form a thiolester, a reaction that is catalyzed by the ubiquitin-activating enzyme (E1) in the presence of ATP. This E2-ubiquitin thiolester complex interacts with an E3 that is bound to a substrate, leading to the transfer of ubiquitin to the substrate protein. Our understanding of the specificity of E2–E3 interactions is incomplete, but available evidence indicates that most individual E3 enzymes use a specific cognate E2 enzyme. In *Saccharomyces cerevisiae*, Ubc7p is the E2 that plays the most prominent role in ERAD, although Ubc1p and, to a lesser extent, Ubc6p can also participate (10–17). Although the mammalian homolog of Ubc7p, HsUbc7/Ube2G2, has been demonstrated to function in ERAD (18–20), it is not clear whether it has a similar general role as its counterpart in yeast. In fact, mammals have many more E2 enzymes than yeast, and the functions of most ubiquitin-conjugating enzymes have not yet been clarified.

The ERAD pathway can be hijacked by certain viruses. In human cells infected with the human CMV, newly synthesized MHC class I heavy chains (HCs) are rapidly dislocated from the ER and degraded by the proteasome, in a manner resembling the dislocation and degradation of misfolded ER proteins (21, 22). Expression of either US2 or US11, two small virally encoded proteins that are inserted into the host ER membrane, is sufficient for this process, indicating that the dislocation of HCs from the ER membrane uses mostly host protein components. The process is best understood for the US11-triggered pathway. It begins with the recognition of HC by US11. US11 probably delivers the substrate to the US11-interacting, multispanning membrane protein Derlin-1, postulated to be a component of a protein-conducting channel (23, 24). On the cytosolic side of the ER membrane, a polyubiquitin chain is attached to a part of the HC that was previously in the ER lumen (22). The polyubiquitin chain is subsequently recognized by an ATPase complex (25–27), consisting of the AAA ATPase p97 and a cofactor (Ufd1p-Npl4p). It is thought that the ATPase complex moves the substrate into the cytosol in a process that requires ATP hydrolysis (4, 25, 27). Much of our current understanding of the US11-dependent dislocation pathway comes from the use of a permeabilized cell system (8) in which astrocytoma cells, stably expressing US11, are permeabilized with digitonin and the cytosol is exchanged or manipulated.

One of the most important steps in the US11-dependent dislocation pathway is polyubiquitination, and yet this process is only poorly understood, particularly because neither the E2 nor the E3

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Abbreviations: HC, heavy chain; ER, endoplasmic reticulum; ERAD, ER-associated degradation; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin protein ligase; f-Ub, Oregon green-labeled ubiquitin.

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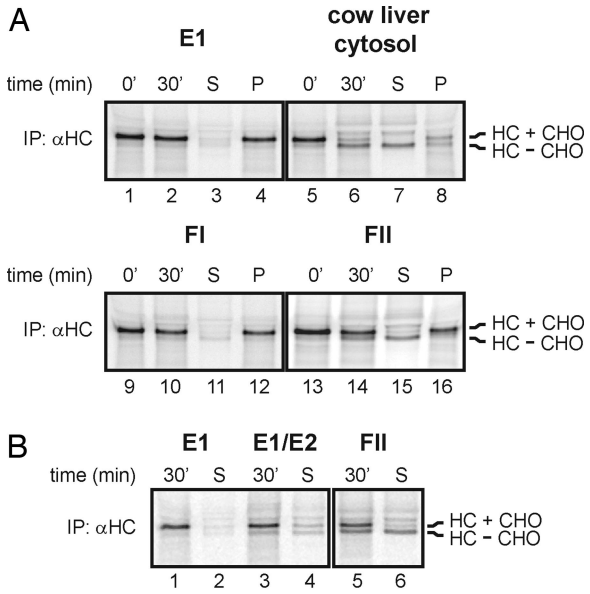

Fig. 1. HC dislocation in permeabilized cells initiated with exogenously added cytosolic proteins. Cells expressing the human cytomegalovirus US11 protein were pulse-labeled with [^{35}S]methione/cysteine, permeabilized, pelleted, and washed to deplete their cytosol. Dislocation reactions were initiated by the addition of ATP together with cytosol or cytosolic proteins as specified. HC immunoprecipitates were analyzed by SDS/PAGE and autoradiography. Radiolabeled proteins corresponding to glycosylated HC and deglycosylated HC are marked as HC+CHO and HC−CHO, respectively. (*A*) Reactions contained either 1 μM E1 (lanes 1–4), cow liver cytosol (lanes 5–8), FI (supplemented with 1 μM E1, lanes 9–12), or FII (lanes 13–16). (*B*) Reactions contained 1 μM E1 (lanes 1 and 2), enriched E1/E2 fraction (lanes 3 and 4), or FII from cow liver cytosol (lanes 5 and 6). Ubiquitin was added to all reactions at 20 μM.

enzymes involved have been identified. In the present study, we have used the permeabilized cell system to identify a ubiquitin-conjugating enzyme (E2-25K) that, together with ubiquitin, ATP, and E1, is sufficient to replace crude cytosol in the HC dislocation assay. We also identify E3 enzymes that activate the activity of E2-25K and are thus candidates for playing a role in the dislocation process.

Results

We previously described a permeabilized cell system to follow US-11 mediated dislocation of MHC class I HCs (8). HC dislocation in such permeabilized cells relies on exogenously added cytosol, which can be manipulated to address the role of cytosolic factors in this reaction. Because polyubiquitinated HC is an intermediate in the dislocation reaction (8, 22, 27), it is reasonable to expect that the required cytosolic factors include proteins involved in HC polyubiquitination. Because most known E2 enzymes are cytosolic proteins, we first concentrated on the identification of an E2 enzyme involved in the dislocation of HCs.

US11-expressing astrocytoma cells were pulse-incubated with [^{35}S]methionine and [^{35}S]cysteine and permeabilized in digitonin, and the cytosol was removed by sedimentation. The cells were resuspended in cow liver cytosol and incubated for an additional 30 min in the presence of unlabeled amino acids. Immunoprecipitation showed that a large fraction of HC was converted into a faster migrating species (Fig. 1*A*, lane 6 versus 5). Previous experiments have shown that the conversion is caused by the removal of a carbohydrate chain from HC, which is catalyzed by a cytosolic N-glycanase, and by deubiquitination of previously polyubiquitinated HC (8, 22). The gel mobility shift is thus an indication of dislocation of HC from the ER lumen to the cytosol. Upon fractionation of the sample by low-speed centrifugation, deglycosylated HC appeared in the supernatant (Fig. 1*A*, lane 7), whereas residual, nondislocated material was found in the pellet (Fig. 1*A*, lane 8). We also observed some discrete species with slower mobility that likely corresponds to ubiquitinated HC (Fig. 1*A*, lane 7). When cytosol-depleted cells were incubated with purified ubiquitin and E1, instead of cytosol, no shift in gel mobility of HC was observed and no material appeared in the supernatant fraction (Fig. 1*A*, lanes 1–4), indicating that these proteins alone are insufficient to support dislocation of HC.

As part of our initial effort to identify the relevant E2, we subdivided cow liver cytosol into two fractions, one that was retained on an anion-exchange gel matrix (FII) and another that was not (FI). Both fractions contain E2 enzymes and FI contains ubiquitin, whereas FII is depleted of ubiquitin (data not shown). When added to permeabilized cells, complementation was found with FII (Fig. 1*A*, lanes 13–16), but not with FI (Fig. 1*A*, lanes 9–12), and combining FI and FII did not increase the efficiency of HC dislocation seen with FII alone (data not shown). Next, we used a well established procedure to further enrich all E2 enzymes from FII (28, 29). The proteins in this fraction were incubated with ubiquitin-coupled gel beads, which led to the binding of all E2 enzymes via a thiolester bond linkage. Elution was performed with a reducing agent that cleaves the thiol bonds (28, 29). Because E1 also forms a thiolester linkage with ubiquitin, it is also present in the eluate. When this E1/E2-enriched fraction was added together with ubiquitin to cytosol-depleted permeabilized cells, dislocation of HC, and the appearance of slower migrating species characteristic of ubiquitinated HC, were observed (Fig. 1*B*, lanes 3 and 4). A portion of HC in the supernatant remained glycosylated (Fig. 1*B*, lane 4), indicating that the E1/E2-enriched fraction may be deficient in N-glycanase activity.

To identify the E2 enzyme, the E1/E2-enriched protein mixture was bound to a Mono-Q column and eluted with a linear salt gradient. Twenty-five fractions were collected and tested for the presence of E2 enzymes by the generation of E2-ubiquitin thiolesters. Twelve fractions contained one or more distinct E2s (data not shown). When these fractions were tested for HC dislocation with cytosol-depleted permeabilized cells, E1, and ubiquitin, fraction 9 was found to be most active (f9, Fig. 2*A*, lanes 5 and 6, and data not shown). When tested for E2-ubiquitin thiolester formation with fluorescently labeled ubiquitin, two E2 enzyme activities were found, the ubiquitin-thiolesters of which migrated on SDS gels with apparent molecular masses of 35 and 27 kDa (Fig. 2*B*, lane 2). The same assay performed with the adjacent fractions indicated that the 35-kDa thiolester was most prominent in fraction 9 (Fig. 2*B*, lanes 1–3), indicating that the corresponding E2 enzyme shows a good correlation with the activity in the dislocation assay. Fraction 9 contains at least four protein species (Fig. 2*B*, lane 5), two of which, labeled as A and C, have molecular masses consistent with E2s that could form ubiquitin-thiolesters of 35 and 27 kDa, respectively. The bands corresponding to proteins A and C were excised and treated with trypsin, and the resulting peptides were subjected to MALDI-TOF analysis. The peptide mass data were used to search the protein database and identified proteins A and C as the ubiquitin-conjugating enzymes E2-25K and Ubc13, respectively. Band D was identified in a similar way as UEV2, an E2 variant that functions with Ubc13 in a heterodimer (30, 31). The identity of these proteins was confirmed by additional MS/MS analyses of two tryptic peptides from each protein, which in all cases yielded fragmentation patterns consistent with the expected peptide sequence. Thus, the two E2 activities seen in f9 are caused by the presence of E2-25K and Ubc13, and E2-25K is the better candidate for being involved in the dislocation of HC.

To directly test a role for E2-25K in HC dislocation, we used purified recombinant protein made in *Escherichia coli*. When purified E2-25K, ubiquitin, and E1 were added to cytosol-depleted permeabilized cells, efficient dislocation of HC was observed (Fig.

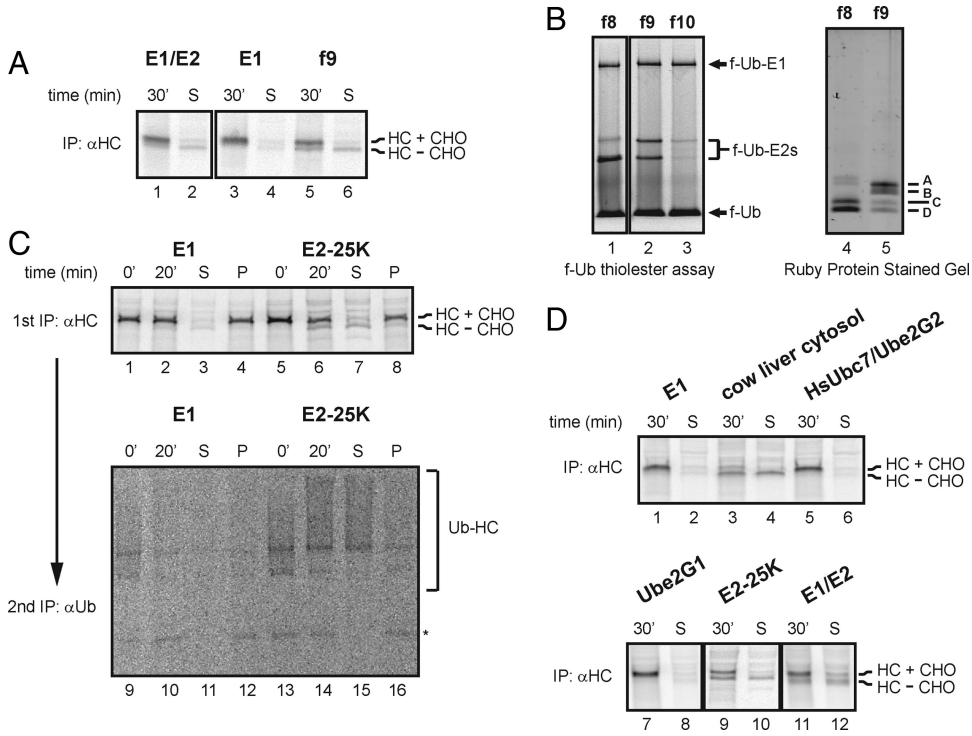

Fig. 2. The E2 activity for HC ubiquitination is supplied by E2-25K. (*A*) SDS-gel analysis of HC immunoprecipitates from reactions using permeabilized cells with added E1/E2-enriched mixture (lanes 1 and 2), 1 μM E1 (lanes 3 and 4), or f9 (supplemented with 1 μM E1, lanes 5 and 6). All reactions were done in the presence of 20 μM ubiquitin. (*B*) Assay of E2-ubiquitin thiolester. In lanes 1–3, fractions (f8–f10) from Mono-Q anion-exchange separation of the E1/E2 mixture were incubated with Oregon green-labeled ubiquitin (f-Ub), E1, and ATP for 10 min. Proteins were separated on SDS-gels and visualized by fluorescence. In lanes 4 and 5, proteins from fractions 8 and 9 were separated on SDS-gels and stained with Sypro Ruby Protein Stain and visualized by fluorescence. (*C*) HC immunoprecipitates from reactions with permeabilized cells supplemented with 1 μM E1 and 20 μM ubiquitin alone, or together with a recombinant E2-25K (5 μM) were either analyzed directly (lanes 1–8) or precipitated further with ubiquitin-specific (αUb) antibodies (lanes 9–16) and then analyzed by SDS/PAGE and autoradiography. The band denoted by * has a mobility similar to HC. (*D*) Comparison of HC dislocation in assays containing E1, cow liver cytosol, E1/E2-enriched fraction, recombinant HsUbc7, Ube2G1, or E2-25K. Reactions contained equivalent amounts of permeabilized cells, and E2s were used at 5 μM, E1 at 1 μM, and ubiquitin at 25 μM. Analysis was done by SDS/PAGE and autoradiography.

2*C*, lanes 5–8). Sequential immunoprecipitation with antibodies against HC and ubiquitin demonstrated directly that HC is ubiquitinated in the presence of added E2-25K (Fig. 2*C*, lanes 13–16), but not with E1 alone (Fig. 2*C*, lanes 9–12). Thus, we conclude that E2-25K can act as the sole E2 for polyubiquitination and subsequent dislocation of HC.

Of the 11 E2s in the yeast *S. cerevisiae*, E2-25K most closely resembles Ubc1p, both in the core UBC domain sequence and in having a C-terminal UBA domain (32, 33). In yeast, Ubc1p and Ubc7p may have overlapping functions in ERAD (10–12, 14, 17). We therefore tested whether the mammalian orthologue of yeast Ubc7p, HsUbc7/Ube2G2, which has been reported to function in ERAD (18–20), can also serve as the only E2 in the HC dislocation assay. In contrast to recombinant E2-25K, neither recombinant HsUbc7/Ube2G2 nor Ube2G1, which bears a close resemblance to Ube2G2, supported HC dislocation (Fig. 2*D*), even though these E2 enzymes were active in a ubiquitin-thiolester formation assay (data not shown). Thus, E2-25K cannot be replaced by HsUbc7/Ube2G2, an enzyme known to function in ERAD. Recombinant human Ubc2b and Ubc3b, two other E2s present in FII, were also unable to support HC dislocation (data not shown), providing additional support that the activity seen with E2-25K is specific. A C-terminal truncation mutant of E2-25K lacking the UBA domain could effectively replace the full-length protein (Fig. 3), indicating that the UBA domain is not essential for the function of E2-25K in HC dislocation.

Next, we wanted to identify potential ubiquitin protein ligase partners of E2-25K, based on the assumption that they would stimulate the activity of the E2. To measure the activity of E2-25K we used the fact that it has the ability to form ubiquitin dimers by transfer of thiolester-linked ubiquitin to free ubiquitin. Additional ubiquitin moieties can then be attached, leading to formation of higher-order ubiquitin oligomers with distributive kinetics (34, 35). Although E2-25K on its own gave rise to only a small amount of ubiquitin dimers and trimers, the addition of cytosol-depleted permeabilized cells greatly stimulated the reaction (Fig. 4*A*, lane 4 versus 2). The addition of ubiquitin aldehyde, an inhibitor of deubiquitinating enzymes, had only a small effect on dimer formation, but boosted the formation of trimers (Fig. 4*A*, lane 3). The addition of E1 alone had also a small effect, presumably because some E2 enzyme is still present in the permeabilized cells (Fig. 4*A*, lanes 5–8). Taken together, these data suggest that a membrane-bound ligase present in permeabilized cells stimulates the activity of exogenously added E2-25K.

Further identification of a potential ligase was based on the previous observation that a similar ubiquitin dimer formation activity of HsUbc7/Ube2G2 is activated by its interaction with the RING domain of its known ubiquitin protein ligase partners Hrd1 or TEB4 (36, 37). We therefore tested whether E2-25K could also be activated by RING domains of ubiquitin protein ligases. The RING domains of several membrane-bound human ubiquitin protein ligases were expressed in *E. coli* and purified. These included the RING domains of the ligases Hrd1, gp78/AMFR, TEB4/MARCH-VI, TRC8, MARCH-I, which activate HsUbc7/Ube2G2 (36, 37), and the RING domains of the ligases MARCH-II, III, IV, VII, VIII, and IX (38), which do not activate HsUbc7/Ube2G2 (unpublished results). When tested with E2-25K, a large stimulation of ubiquitin dimer formation was seen with the RING domains of gp78/AMFR or MARCH-VII (Fig. 4*B*, lanes 4 and 8).

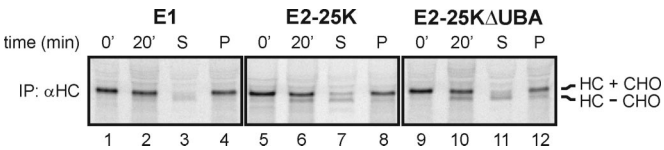

Fig. 3. E2-25K does not require its UBA domain for dislocation of HC. Reactions containing radiolabeled permeabilized cells were supplemented with 1 μM E1, 25 μM ubiquitin alone, or in the presence of 5 μM either wild-type E2-25K or the UBA domain deletion mutant E2-25KΔUBA (described in *Materials and Methods*). HC was recovered by immunoprecipitation with anti-HC (αHC). Analysis was done by SDS/PAGE and autoradiography.

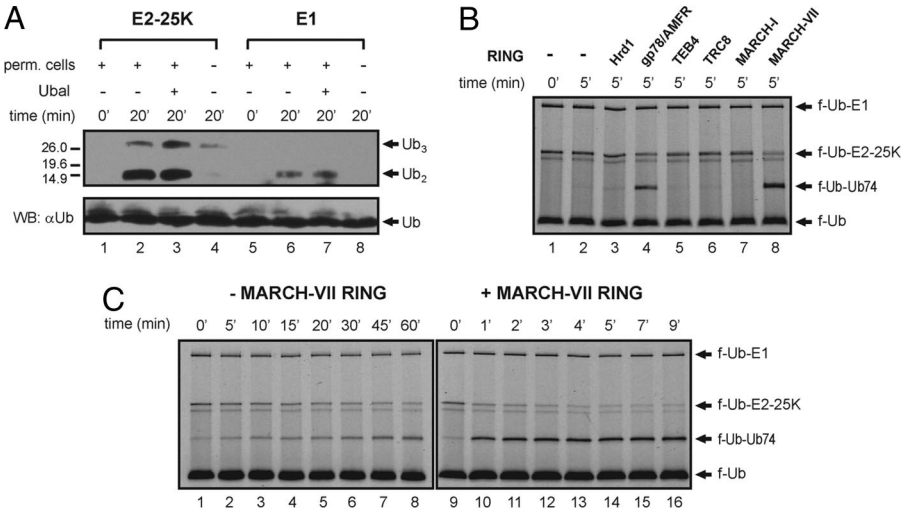

Fig. 4. Activation of an intrinsic ubiquitin dimer formation activity in E2-25K. (*A*) Immunoblot showing ubiquitin oligomer formation by E2-25K. Reaction mixtures containing 1 μM E1 and 100 μM ubiquitin with or without 5 μM E2-25K were added to permeabilized cells and incubated at 37°C for 20 min. Parallel reactions were also carried out in the absence of permeabilized cells. Where indicated, permeabilized cells were pretreated with ubiquitin C-terminal aldehyde (Ubal). (*B*) Ubiquitin dimer assay in the absence and presence of the specified RING domains. E2-25K-f-Ub thiolester was prepared under single turnover conditions as described in *Materials and Methods*. Preformed E2-25K-f-Ub was then incubated for 5 min at 4°C without and with 15 μM indicated RING domain and 10 μM Ub74. Analysis was done by SDS-gel separation of proteins and visualized by fluorescence. (*C*) Time course of ubiquitin dimer formation was carried out with preformed E2-ubiquitin thiolester as described in *B* using 25 μM Ub74, in the absence or presence of 5 μM RING domain from MARCH-VII/axotrophin.

Representative time courses of this reaction with and without the MARCH-VII/axotrophin RING domain are shown in Fig. 4*C*. The other RING domains, including TEB4, the proposed homolog of Ssm4p/Doa10p, which has also been implicated in ERAD (39) showed no significant activity (Fig. 4*B*, lanes 3 and 5–7, and data not shown). Thus, E2-25K interacts specifically with the RING domains of gp78/AMFR and MARCH-VII. Although at least two different E2s (E2-25K and HsUbc7/Ube2G2) are activated by gp78/AMFR, E2-25K is the only known E2 that interacts with MARCH-VII. The two identified ligases are prime candidates to be involved in the US11-triggered dislocation of HC.

Discussion

We have identified a ubiquitin-conjugating enzyme, E2-25K, that together with ubiquitin and the ubiquitin-activating enzyme, can replace crude cytosol in generating polyubiquitinated HC, allowing its subsequent dislocation from the ER into the cytosol. Our results suggest that this E2 enzyme is involved in the US11-triggered degradation of MHC class I HCs, but further experiments are required to test its function *in vivo*. E2-25K was first discovered by the Pickart laboratory (33, 34) by tracing an intrinsic activity of this enzyme that allows the formation of ubiquitin dimers linked through Lys-48 (K48). Although synthetic K48-specific polyubiquitin chains made with the help of this enzyme have been instrumental in our understanding of how polyubiquitinated proteins are recognized by the proteasome (40–42), a biological function for this enzyme had not been established previously to our knowledge. The formation of K48-linked polyubiquitin chains by E2-25K is consistent with our previous observation that these types of chains are required for interaction with the p97 ATPase complex that functions downstream by moving polyubiquitinated HC into the cytosol (25, 26). Most of the ATPase complex is tightly bound to ER membranes and thus present in cytosol-depleted permeabilized cells, explaining why purified E2, E1, and ubiquitin can satisfy the cytosol requirement in our assay. Although we have shown that purified E2-25K is sufficient to replace crude cytosol, we cannot exclude the possibility that other E2 enzymes with redundant function were inefficiently recovered during the purification procedure. Also, it is possible that E2 enzymes are involved that are already associated with the ER membrane. In yeast, ERAD mediated by Doa10p requires Ubc6p, an E2 enzyme that is anchored to the ER membrane via a C-terminal transmembrane domain and Ubc7p that is recruited to the ER membrane via binding to Cue1p (39). Thus, it remains to be determined whether HC ubiquitination may have an additional requirement for a membrane-associated E2.

The possibility that E2-25K functions in ERAD is further suggested by its resemblance to *S. cerevisiae* Ubc1p, which among the 11 E2 enzymes in yeast is most similar in sequence to E2-25K. This similarity includes the presence of a UBA domain not found in other members of this family. However, it is not known whether E2-25K provides the equivalent function(s) in mammals or whether E2-25K can complement a UBC1 deletion in *S. cerevisiae*. In yeast, Ubc1p functions with Hrd1p, an ER-resident ubiquitin protein ligase that mediates the degradation of ER proteins with misfolded luminal or intramembrane domains (12, 14, 43). Ubc1p appears to share its function with Ubc7p, as either of these two enzymes can interact with the RING domain of Hrd1p (12, 14, 17, 43), and HRD1-deletion phenotypes are recapitulated in a UBC1/UBC7 double-deletion mutant, but only partially in UBC1 or UBC7 single-deletion mutants (17). Similar to the dual utilization of Ubc1p and Ubc7p by Hrd1p, we have observed that both E2-25K and HsUbc7/Ube2G2 can be activated by the RING domain of gp78/AMFR, a ligase that is sequence-related to Hrd1p. Previous studies have indeed implicated gp78/AMFR and HsUbc7 in the regulated degradation of HMG-CoA reductase and the destruction of unassembled T cell receptor (20, 44, 45). Our results thus raise the possibility that, as in yeast, E2-25K might function interchangeably with HsUbc7 in these ERAD events. Many other aspects of the ERAD pathways are conserved between yeast and mammals, but the mammalian system is clearly more complex, with often more than one homolog for a given yeast component. For example, there are two mammalian homologs of yeast Ubc7p (HsUbc7/Ube2G2 and Ube2G1) and yeast Hrd1p (Hrd1 and gp78/AMFR), and it is possible that they function in the polyubiquitination of distinct substrates.

E2-25K cannot be replaced by HsUbc7/Ube2G2 in its function in US11-triggered HC dislocation, but our data do not exclude that other E2s could also function in HC polyubiquitination; the residual levels of E2 in cytosol-depleted permeabilized cells make the assay rather insensitive and it is therefore possible that other fractions in our purification steps also contain some activity. Nevertheless, the homologs of yeast Ubc1p and Ubc7p are certainly the most obvious candidates for a function in ERAD and HC dislocation. It is therefore surprising that only the Ubc1p homolog E2-25K, and not the Ubc7p homolog, functions in HC polyubiquitination. Apparently, the ubiquitin protein ligase involved in HC polyubiquitination is more selective than gp78/AMFR, which interacts with both E2-25K and

the Ubc7p homolog. For this reason, gp78/AMFR is unlikely to be the relevant E3 in HC ubiquitination. The only other tested RING domain that activated E2-25K was the one derived from MARCH-VII/axotrophin, and this RING domain did not activate HsUbc7/Ube2G2 (unpublished result). MARCH-VII is therefore a prime candidate to be the ubiquitin protein ligase involved in the polyubiquitination of HC. MARCH-VII has two putative transmembrane sequences, but its subcellular localization is unknown. Whether this ligase has a function in US11-triggered HC dislocation remains to be determined.

Materials and Methods

Pulse–Chase Analysis with Permeabilized Cells. Control and US11-expressing U373-MG astrocytoma cells (46) were cultured as described (21). The cells were detached from tissue culture flasks with trypsin and incubated in suspension in methionine- and cysteine-free DMEM for 1 h at 37°C. Cells were then centrifuged at 1,000 × *g* for 5 min and resuspended at 1×10^7/ml. They were pulse-labeled for 3–5 min at 37°C in 290 μCi/ml [^{35}S]methionine and -cysteine (^{35}S-Protein Express Labeling Mix; New England Nuclear, Wellesley, MA), after which all procedures were carried out at 4°C. The cells were centrifuged for 15 s at 18,000 × *g* in a microfuge and resuspended at the same cell density in PBS supplemented with 0.9 mM $CaCl_2$. They were then centrifuged as described, resuspended at 1.6×10^7 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 EGTA) containing 0.04% digitonin (Merck, Whitehouse Station, NJ, purified as described in ref. 47), and incubated on ice for 10 min with gentle agitation. The digitonin-permeabilized cells were pelleted by centrifugation for 15 min at 18,000 × *g* in a microfuge, resuspended at the same density in PB without digitonin, and pelleted again by centrifugation as described.

The chase reaction was initiated by resuspending the permeabilized cell pellets in a buffer of 25 mM Tris·HCl, pH 7.6, containing 10 μM proteasome inhibitor PS-341, 1 mM ATP, 1 mM magnesium acetate, an ATP-regenerating system (48), and other specified protein components. The samples were incubated at 37°C, and aliquots were withdrawn at specified time points. Withdrawn samples were divided into two equal parts. One part was placed directly into NET lysis buffer, consisting of 0.5% Nonidet P-40 (Igepal; CA-630; Sigma, St. Louis, MO), 50 mM Tris·HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, a mammalian protease inhibitor mix (Sigma), and 0.2 mg/ml BSA. The other part was centrifuged for 15 min at 18,000 × *g* in a microfuge, to obtain supernatant and pellet fractions, which were then separately placed into NET lysis buffer. Lysates were made by agitation on a rotator for 20 min at 4°C and subsequently centrifuged in a microfuge at 18,000 × *g* for 10 min. The resulting supernatant was used for immunoprecipitation after SDS and DTT were added to final concentrations of 0.1% and 0.2 mM, respectively. The lysates made from samples corresponded to 1–1.5×10^6 cell equivalents. Immunoprecipitations were carried out as described (22).

Expression and Purification of Recombinant Proteins. E2-25K was either expressed as a GST fusion (33, 49) or a nontagged protein (49, 50) and purified accordingly. For the GST fusion, the GST moiety was removed by thrombin cleavage, which left behind a 13-aa sequence of RRASVGSHMPMGD fused to the N terminus of E2-25K. The UBA domain deletion mutant of E2-25K (E2-25KΔUBA) was created by changing the codon for residue 156 into a stop codon, expressed as a GST fusion, and purified with the GST removed. HsUbc7/Ube2G2 was obtained by expression as a tobacco etch virus protease-cleavable polyHis-tagged protein and purified as described (51). The purified HsUbc7 contains an extra two-residue sequence of Gly-His at its N terminus. HsUbe2G1 was expressed as a GST fusion and purified with the GST moiety removed but with an extra two-residue sequence of Gly-Ser at its N terminus.

RING domain constructs for Hrd1 and gp78/AMFR were expressed as histidine-tagged fusions. The coding sequences for the RING domains were inserted between the NdeI and HindIII sites of the pT7 plasmid. The inserted sequence for Hrd1 encodes residues 272–342 and encodes residues 322–394 for gp78/AMFR. All other proteins were expressed as GST fusions, where the RING domain coding sequences were inserted between the BamH1 and EcoR1 sites in pGEX-4T1. The inserted sequence encodes residues 529–599 for TRC8, 46–102 for MARCH-I, 47–120 for MARCH-II, 52–127 for MARCH-III, 145–221 for MARCH-IV, 1–80 for MARCH V, 537–619 for MARCH-VII, 62–137 for MARCH-VIII, and 91–168 for MARCH IX. The GST-TEB4/MARCH-VI fusion was as described (37).

A truncated ubiquitin (Ub74) that lacks the C-terminal Gly-Gly sequence was obtained by expressing the human sequence that encodes the first 74 residues of the protein.

Fractionation of Cow Liver Cytosol. FI and FII were isolated from cow liver cytosol by using a procedure described for reticulocyte lysate (28). Briefly, 50 ml of cow liver cytosol (1.5 g protein) in 25 mM Tris·HCl, pH 7.6 was absorbed onto a 200 ml Q-Sepharose (Amersham Pharmacia, Piscataway, NJ) column. The pass-through proteins were precipitated by adjusting the solution to contain 90% ammonium sulfate to yield FI. Bound proteins were eluted with 25 mM Tris·HCl (pH 7.6) and 0.6 M sodium chloride and precipitated similarly to yield FII. The precipitated proteins were dialyzed against 25 mM Tris·HCl, pH 7.6, containing 0.1 mM DTT and stored at −80°C until used. FI contained 72 mg/ml and FII contained 31 mg/ml protein.

Ubiquitin Affinity Column Chromatography. E1 and E2 were obtained from FII as described (28, 29). Ubiquitin-coupled gel beads were obtained by coupling purified ubiquitin (40 mg) to 1.5 ml of activated CH-Sepharose (Amersham Pharmacia) according to the manufacturer's protocol. To obtain an E1 and E2-enriched fraction from FII, 50 mg of FII proteins, supplemented with 5 mM ATP and 10 mM magnesium chloride, was applied to the ubiquitin-coupled gel beads that were previously equilibrated with 25 mM Tris·HCl, pH 7.6, containing 5 mM ATP and 10 mM magnesium chloride. After removal of unbound proteins by washing with equilibration buffer, the gel beads were treated with 25 mM Tris·HCl (pH 9) and 25 mM DTT to facilitate the cleavage of thiolester bond that leads to release of E1 and E2 enzymes from the gel beads. Eluted proteins from this condition were dialyzed against 25 mM Tris·HCl (pH 7.6) and 1 mM DTT and stored at −80°C until use.

E2-Ubiquitin-Thiolester Assays. *In vitro* assays were carried out at room temperature in a reaction mixture containing 25 mM Tris·HCl (pH 7.6), 5 mM ATP, 10 mM magnesium chloride, E1 enzyme (0.1 μM), and Oregon green-labeled ubiquitin (f-Ub) (22). Reactions were carried out for 5 min, and proteins in the reaction mixture were separated by SDS/PAGE under nonreducing conditions. Fluorescence of the Oregon green label was visualized by a fluorescence imager (Fluorimager 595, Molecular Dynamics, Piscataway, NJ).

Ubiquitin Dimer Assays. Reactions were carried out under single-turnover conditions, where ubiquitin in the preformed ubiquitin-E2-25K thiolester complex is transferred to a free ubiquitin in the reaction mixture (34). The thiolester complex was obtained by incubating 4 μM E2-25K for 5 min at 25°C in a mixture containing 50 mM Tris·HCl (pH 7.6), 1 mM ATP, 1 mM magnesium chloride, E1 enzyme (0.1 μM), and f-Ub (≈1 μM). At the end of the incubation period, 10 mM EDTA was added

to chelate magnesium to block further thiolester formation. Dimer formation reactions were initiated upon addition of the preformed E2-thiolester to a mixture containing 25 mM Tris·HCl (pH7.6) and 25 mM C-terminally truncated ubiquitin (Ub74), and the indicated E3 RING domains. The mixtures were incubated at 4°C for 5 min (Fig. 4*B*), or during a time course for the times indicated (Fig. 4*C*). The reaction was stopped by addition of sample buffer without reducing agents. Samples were analyzed by SDS/PAGE and a fluorimager.

Mass Spectrometry. Mass spectrometry was done at the Molecular Core facility at the Pennsylvania State University College of Medicine on a fee-for-service basis. Database searches were performed with the program MASCOT (52).

Materials. Human ubiquitin was expressed and purified from the bacterial strain AR58 (53). Oregon green-labeled ubiquitin (8) and ubiquitin-aldehyde were synthesized as described (54, 55). E1 was purified from rabbit reticulocyte lysate (29) as described. Cow liver cytosol was prepared as described (8). Anti-HC serum and antibodies against bovine ubiquitin were as described (22).

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