Degradation of proteins from the endoplasmic reticulum is fundamental to quality control within the secretory pathway, serves as a way of regulating levels of crucial proteins, and is utilized by viruses to enhance pathogenesis. In yeast two ubiquitin-conjugating enzymes (E2s), UBC6p and UBC7p are implicated in this process. We now report the characterization of murine homologs of these E2s. MmUBC6 is an integral membrane protein that is anchored via its hydrophobic C-terminal tail to the endoplasmic reticulum. MmUBC7, which is not an integral membrane protein, shows significant endoplasmic reticulum colocalization with MmUBC6. Overexpression of catalytically inactive MmUBC7 significantly delayed degradation from the endoplasmic reticulum of two T cell antigen receptor subunits, α and CD3-β, and suggests a role for the ubiquitin conjugating system at the initiation of retrograde movement from the endoplasmic reticulum. These findings also implicate, for the first time, a specific E2 in degradation from the endoplasmic reticulum in mammalian cells.

In eukaryotes, a primary means by which proteins are targeted for degradation is by their modification with chains of ubiquitin (Ub). Ubiquitinated proteins are recognized and degraded by the multicatalytic 26 S proteasome. Attachment of Ub to proteins involves a process in which one of a number of different Ub-conjugating enzymes (UBCs or E2s) accept Ub from activated E1 enzyme in a transthiolation reaction and subsequently catalyze the formation of an isopeptide bond between Ub and substrate, either with or without the involvement of an Ub-protein ligase (E3) (1). Proteasomal degradation is not limited to proteins native to the nucleus and cytosol where proteasomes reside. Many transmembrane and lumenal proteins of the secretory pathway are degraded from the endoplasmic reticulum of two T cell antigen receptor subunits, α and CD3-β, and suggests a role for the ubiquitin conjugating system at the initiation of retrograde movement from the endoplasmic reticulum. These findings also implicate, for the first time, a specific E2 in degradation from the endoplasmic reticulum in mammalian cells.

In yeast, a number of ERAD substrates are multiubiquitinated, examples include mutant forms of Sec61p (6) and carboxypeptidase Y (CPY*) (7) as well as HMGCoA-reductase (3). Genetic analysis has implicated two yeast E2s, UBC6p and UBC7p, in ERAD. Deletion of UBC6 and UBC7 stabilizes mutated Sec61p, Sss1p, CPY, Pdr5, and uracil permease (6–9). Genetic analysis has implicated two yeast E2s, UBC6p and UBC7p, in ERAD. Deletion of UBC6 and UBC7 stabilizes mutated Sec61p, Sss1p, CPY, Pdr5, and uracil permease (6–9). UBC6p is a C-terminal anchored membrane protein whose catalytic site faces the cytosol (10). Unlike UBC6p, UBC7p lacks a membrane anchor but associates with an ER-bound protein, Cue1p (11).

In mammalian cells ERAD substrates such as cystic fibrosis transmembrane conductance regulator and apoB are ubiquitinated in a cotranslational fashion in vitro (12, 13). Subunits of the T cell antigen receptor (TCR), when not assembled into complexes capable of exiting the ER, are also degraded from the ER. In T lymphocytes mult ubiquitinated forms of TCR-α and the TCR CD3-β subunit are associated with the ER membrane, suggesting that their ubiquitination occurs while still membrane-bound (14). In initial studies on ERAD of major histocompatibility complex class I proteins, evidence for ubiquitination was lacking. However, more recent analyses have provided evidence for ubiquitinated major histocompatibility complex class I molecules as degradation intermediates (15). Collectively, these finding suggest that in mammals, as in yeast, components of the Ub conjugating machinery functionally interact with substrates at the ER membrane.

Despite a clear role for ERAD in mammals, no specific E2s have been implicated in this process. We now report characterization of mammalian E2s homologous to yeast UBC6p and UBC7p, establish that these proteins are ER membrane pro-
teins, and provide evidence that a murine UBC7p homolog, MmUBC7, plays a role in the degradation of unassembled TCR subunits from the ER.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Cos-7 (number CRL1651; American Tissue Culture Collection, Manassas, VA) and HEK-293 (number CRL1573; American Tissue Culture Collection) cells were maintained in complete Dulbecco’s modified Eagle’s medium and transfected using the calcium-phosphate method as described (16). Anti-CD3-α (R9) (17); anti-ubiquitin (18); and anti-TCR-α, H2S (19) have all been described. Anti-TCR-β (12CA5) and anti-Myc (9E10) monoclonal antibodies were from StressGen Biotech. (Victoria, Canada). Anti-MDM2 was from Oncogene Science (Cambridge, MA).

Plasmids—Lysineless TCR-α (K–TCR-α/pCDNA3.1) was a gift from Dr. Ron Kostel. Wild-type β2M TCR-α in pCDM8 (Invitrogen, Carlsbad, CA) and CD3-β in pCI (Promega, Madison, WI) were obtained from Dr. Juan Bonifacino. MDM2/pCINeo was a gift from Dr. Shengyuan Fang. GenBank™ expressed sequence tag data bases were searched using the amino acid sequences of yeast UBC6p and UBC7p. Murine cDNA clones that were homologous to yeast sequences (referred to as MmUBC6 and MmUBC7) were obtained and sequenced. MmUBC6 was cloned into pCI digested with the same enzymes and tagged with HA epitope at the N-terminal by polymerase chain reaction using the primers 5′-ATAGAATTCCTACCATGCCGTACCATACGTCGACGGAGGAGGATTTAGAAATAC-3′ and 5′-GCCAATCTCATAAGGAGTCATC-3′. The polymerase chain reaction product was digested with EcoRI and SacI and cloned into pCI digested with the same enzymes. The construct was confirmed by sequencing. To generate MmUBC6 lacking the C-terminal tail, nucleotides encoding the last 52 amino acids were removed by restriction enzyme digestion, and the remainder of the cDNA was subcloned into pCDNA3 (Invitrogen, Carlsbad, CA). MmUBC7 was tagged with Myc epitope at the N terminus using primers 5′-ATAGAATTCCTACCATGCCGTACCATACGTCGACGGAGGAGGATTTAGAAATAC-3′ and 5′-AACGACGGCCAGCTGCCAAGGATTTAGAAATAC-3′. The polymerase chain reaction product was digested with EcoRI and NotI and cloned into pCDNA3. The construct was confirmed by sequencing. Site-directed point mutations were created using QuickChange Mutagenesis kit (Stratagene, La Jolla, CA). The mutations were confirmed by sequencing.

MmUBC6 and MmUBC7 were also cloned into pGEX-KG (20) to generate N-terminal glutathione S-transferase fusions. For some experiments, glutathione S-transferase moieties were cleaved by thrombin treatment and residual thrombin was removed by benzamidine-Sepharose beads according to the manufacturer’s protocol (Amersham Pharmacia Biotech).

In Vitro Transcription and Translation—Coupled in vitro transcription and translation was done in rabbit reticulocyte lysates (Promega, Madison, WI) in the presence or absence of canine pancreas microsomal membranes. Membranes were isolated by centrifugation at 100,000 g for 30 min in an air centrifuge. For urea treatment, the membranes were resuspended in 2.5 mM urea for 15 min at room temperature followed by centrifugation and separation of supernatant and intermitogen. The supernatant was precipitated with 10% trichloroacetic acid and the pellet resuspended in 0.1 N NaOH.

Immunofluorescence Analysis—Cos-7 cells were grown on coverslips and transfected using Superfect (Qiagen, Valencia, CA) according to manufacturer’s protocol. Cells were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min then washed twice with PBS. This was followed by incubation with 12CA5 or 9E10 antibody diluted 1:5 in PBS containing 0.1% saponin and 0.1% bovine serum albumin for 30 min followed by washing with PBS and incubation with secondary antibody coupled to Cy3. For double staining, coverslips were then incubated with second primary antibody conjugated to biotin followed by staining with streptavidin coupled to Alexa 488. Images were obtained on a Leica confocal microscope using 63× and 100× objectives. The 488-nm line of the Argon laser and 568-nm line of the Krypton laser were used to excite Alexa 488 and Cy3, respectively. Emission was collected between 520 and 530 nm for Alexa 488 and between 610 and 700 nm for Cy3.

Pulse-Chase Analysis—HEK-293 cells were transfected using calcium-phosphate method with 2.5 μg of 2B4 TCR-α/pCDMS and 5 μg each of either wild type or mutant HA-MmUBC6 or Myc-MmUBC7 or both together. The total amount of plasmid was equalized with pCDNA3. Cells were harvested 36 h post-transfection and incubated for 45 min in methionine-free medium and then labeled for 20 min with 25 μCi of [35S]methionine/ml (ICN Biomedicals, Costa Mesa, CA). Cells were then washed twice at 4 °C with complete medium followed by incubation in complete medium at 37 °C. Cells were harvested at indicated times, and immunoprecipitations were performed as described below followed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Immunoprecipitations and Western Blotting—Cells were lysed in Triton X-100 lysis buffer containing protease inhibitors as described (14). Immunoprecipitations were carried out at 4 °C using protein A-Sepharose beads prebound to indicated antibodies. Beads were washed with 50 mM Tris, pH 7.4, 300 mM NaCl, 0.1% Triton X-100. In some cases 0.05% SDS was included in the wash buffer. SDS-polyacrylamide gel electrophoresis and Western transfer were done according to standard protocols. Blots were developed using either 125I-labeled protein A or chemiluminescence (Pierce).

Proteinase K Protection and Subcellular Fractionation—Cells were lysed in triethanolamine buffer and broken as described (14). Unbroken cells and nuclei were removed by centrifugation at 1000 x g for 5 min. The supernatant was divided in two, and one half was treated with proteinase K as described (14) Samples were centrifuged at 100,000 g for 45 min at 4 °C. Pellets were washed once with triethanolamine buffer and solubilized in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% digitonin). To the supernatant, 2 x digitonin lysis buffer was added. All samples were centrifuged again at 10,000 x g for 10 min, and the supernatants were immunoprecipitated with H28 antibody and analyzed by gel electrophoresis.

N-Glycanase Treatment—Treatment with N-glycanase (PNGase F, New England Biolabs Inc., Beverly, MA) was carried out as described by the manufacturer.

RESULTS

Sequence Analysis of MmUBC6—To identify mammalian orthologs of yeast UBC6p, the GenBank™ expressed sequence tag data base was searched and homologous murine cDNAs obtained and sequenced. One such clone included a putative start codon that was in a good context for translation initiation (21) with a stop codon 5′ to this site in the same reading frame (GenBank™ accession number AF296656). The open reading frame predicts a protein of 262 amino acids, 12 amino acids more than UBC6p (Fig. 1). This putative protein is referred to hereafter as MmUBC6 for the murine homolog of yeast UBC6p based on the convention that E2s are named for the corresponding Saccharomyces cerevisiae member preceded by a two-letter genus/species abbreviation. Homology with UBC6p is most striking in the region surrounding the core E2 domain (amino acids 8–166 of UBC6p), where there is 59% identity and 72% similarity. However, beyond amino acid 166 of the mouse protein there is less than 11% identity. MmUBC6 was determined to be an active E2 by the ability of recombinant MmUBC6 to form thiol ester bonds with Ub in the presence of E1 (data not shown). This activity was abolished by mutation of the predicted active site cysteine (amino acid 94).

UBC6p is unique among E2s in that it is a type IV membrane protein, defined as such by its C-terminal membrane anchor that allows for its ER membrane insertion in yeast (10) and when ectopically expressed in mammalian cells (22). MmUBC6 similarly has a C-terminal hydrophobic domain, although it is highly divergent from yeast UBC6p. Hydrophilicity analysis using MacVector (Oxford Molecular Group, Oxford, UK) and GGC (Genetics Computer Group Inc., Madison, WI) programs suggests that this domain begins at amino acid 228 and extends at least to amino acid 249. Notably, a domain at amino acid 245–247 that allows for its ER membrane insertion in yeast (10) and similar has a C-terminal hydrophobic domain, although it is most striking in the region surrounding the core E2 domain (amino acids 8–166 of UBC6p), where there is 59% identity and 72% similarity. However, beyond amino acid 166 of the mouse protein there is less than 11% identity. MmUBC6 was determined to be an active E2 by the ability of recombinant MmUBC6 to form thiol ester bonds with Ub in the presence of E1 (data not shown). This activity was abolished by mutation of the predicted active site cysteine (amino acid 94).

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Membrane Association and Subcellular Localization of MmUBC6—To determine whether MmUBC6 is a membrane protein, it was translated in rabbit reticulocyte lysate in the presence of canine microsomes. This yielded a ~31-kDa protein (Fig. 2), of which substantially more than 50% was consistently found in the membrane fraction (Fig. 2A, compare lanes 1 and 2). Membrane-associated MmUBC6 was resistant to urea extraction (Fig. 2A, lanes 3 and 4) and released into the soluble fraction upon extraction with Triton X-100 (Fig. 2A, lanes 5 and 6). A truncation lacking the C-terminal hydrophobic domain (MmUBC6d) partitioned almost entirely into the soluble fraction (Fig. 2A, lanes 7 and 8). Thus, MmUBC6 is also a C-terminal anchored, integral membrane protein. Other C-terminal anchored membrane proteins have been reported to be inserted in membranes post-translationally. To determine whether this is the case for MmUBC6, it was translated in vitro without microsomes and then assessed for membrane insertion by incubation with canine microsomes followed by separation of membrane and soluble fractions by centrifugation. When membranes were added, ~40% of MmUBC6 partitioned into the membrane fraction (Fig. 2B, c and d) and was largely resistant to urea extraction (Fig. 2B, e and f). In contrast MmUBC6d was almost entirely recovered in the soluble fraction (Fig. 2B). When ectopically expressed in mammalian cells, increasing the C-terminal hydrophobic domain of yeast UBC6p from 17 to 21 amino acids results in a redistribution along the secretory pathway from the ER to the Golgi (22). The predicted hydrophobic domain of MmUBC6 is longer than its yeast counterpart. However, its subcellular localization by immunofluorescence revealed a lacelike ER pattern (Fig. 3, C and G) and colocalization with UBC6p (Fig. 3, D–F). Thus, MmUBC6 is also an ER membrane protein.

Identification of a Murine Homolog of UBC7p—Rat and human homologs of UBC7p have been reported (24, 25); however, no evidence for physical or functional association with the ER has been demonstrated. A murine UBC7p homolog was identified in the expressed sequence tag data base that encodes another core E2, UbcH5B (26) did not bind to membranes (Fig. 4A), whereas membrane association of MmUBC7 was undertaken by transient expression of Myc-

![Fig. 1. Amino acid sequence comparison of MmUBC6 with S. cerevisiae UBC6p. Dark gray boxes represent identical amino acids, and light gray boxes show similarity. Active site Cys is indicated with an asterisk. Hydrophobic C-terminal domains are underlined.](http://www.jbc.org/)

![Fig. 2. MmUBC6 is a type IV membrane protein. A, MmUBC6 and MmUBC6d were translated in vitro using rabbit reticulocyte lysate and microsomal membranes. Translated protein was fractionated into soluble (S) and pellet (P) fractions (lanes 1, 2, 7, and 8) by centrifugation. The pellet was further extracted with 2.5 M urea (lanes 3 and 4) or Triton X-100 (lanes 5 and 6) and again separated into soluble and pellet fractions. B, MmUBC6 and MmUBC6d were translated in vitro in the absence of membranes followed by fractionation into soluble (S) and membrane (P) fractions either without incubation with membranes (a and b) or after incubation with membranes (c and d). A duplicate of the pellet from d was extracted with 2.5 M urea and separated in soluble and pellet fractions. MmUBC6d was treated similar to samples in c and d.](http://www.jbc.org/)
epitope-tagged MmUBC7. MmUBC7 was found both in the cytosolic and microsomal membrane fractions (Fig. 4B, upper panel, lanes 1 and 3). In contrast all of the anti-UbcH5B immunoreactivity was in the cytosolic fraction (Fig. 4B, lower panel, lanes 1 and 2).

*MmUBC7 Colocalizes with MmUBC6*—When evaluated by confocal immunofluorescence microscopy, MmUBC7 appears to be diffusely expressed, but a discrete underlying ER pattern is evident (Fig. 5A). To confirm this ER association, it was coexposed with MmUBC6. Fig. 5 (B–D) shows confocal images from the same cell expressing both HA-MmUBC6 (5B) and Myc-MmUBC7 (5C). A significant colocalization of Myc-MmUBC7 with HA-MmUBC6 to the ER was observed when (5B) and (5C) were overlaid (Fig. 5D, yellow shows colocalization).

**Overexpression of Inactive MmUBC7 Inhibits Degradation of T Cell Receptor Subunits**—Previous studies have established that ERAD of TCR-α, a type I transmembrane protein with a short cytoplasmic tail of 5 amino acids, is dependent on proteasome function. To evaluate whether mammalian homologs of UBC6p and UBC7p are involved in targeting TCR-α for degradation, catalytically inactive forms of these E2s were coexpressed with TCR-α in HEK-293 cells. Levels of TCR-α were evaluated by immunoblotting with anti-TCR-α. Although overexpression of a mutant of MmUBC6 in which the active site Cys was converted to Ser (C94SMmUBC6) did not substantially affect TCR-α levels, the analogous catalytically inactive MmUBC7 (C94SMmUBC7) resulted in marked increase in TCR-α levels (Fig. 6, A and B). Coexpression of the two inactive E2s did not result in a further increase (Fig. 6A). In contrast, overexpression of inactive MmUBC7 did not result in accumulation of Mdm2, which is a non-ER protein that is ubiquitinated and degraded by proteasomes (Fig. 6C). Neither wild type MmUBC6 nor MmUBC7 had any significant or reproducible effect on TCR-α (Fig. 6, A and B).

To confirm that the effect observed with overexpression of C94SMmUBC7 was due to inhibition of degradation, TCR-α half-life was directly determined by pulse-chase metabolic labeling. TCR-α consistently exhibited a 45–65% increase in half-life when C94SMmUBC7 was coexpressed (Table I). Concomitant overexpression of inactive MmUBC6 did not further increase TCR-α survival.

**Majority of TCR-α Is Largely Membrane-bound and Proteinase K-resistant**—Previous studies in non-T cells have suggested that when proteasome function is inhibited, a significant amount of the accumulated TCR-α has undergone retrotranslocation from the ER to the cytosol accompanied by deglycosylation (29, 30). To determine the location of TCR-α that accumulates with inactive MmUBC7, cells expressing TCR-α were subjected to proteinase K (PnK) digestion followed by separation of cytosolic and membrane fractions (Fig. 7). When inactive MmUBC7 was coexpressed, increased amount of TCR-α was seen (compare lanes 1–4, 9–12, and 17–21). All of this accumulated TCR-α was found in the membrane fraction and was resistant to PnK digestion and therefore has not undergone retrotranslocation through the ER membrane. When proteasome function was inhibited (lanes 5–8, 13–16, and 21–24) levels of accumulated TCR-α increased, still the large majority of the immunoreactive material was found in the membrane fraction resistant to PnK. However, a small fraction of the accumulated TCR-α was PnK-sensitive and included full-length and more rapidly migrating species (open arrow) that represent deglycosylated forms (see below and Fig. 7B) in the cytosolic fraction as well as similar rapidly migrating species in the membrane fractions. Although these PnK-sensitive forms were observed in all proteasome-treated samples, when compared with the levels of full-length PnK-resistant TCR-α, ret-
rotranslocated forms were found at a proportionally lower level in cells expressing inactive MmUBC7. The findings that inactive MmUBC7 results in the accumulation of fully protected membrane-associated TCR-α and that there is a proportional decrease in cytoplasmically disposed species when proteasome function is inhibited implicates MmUBC7 in playing a role in ERAD prior to retrograde movement through the ER membrane.

As already noted, the faster migrating species observed when proteasome function is inhibited are consistent with previous reports for TCR-α where cleavage of N-linked oligosaccharides is observed concomitant with retrotranslocation to the cytosol.

**TABLE I**

| Experiment | C94S MmUBC6 | C89S MmUBC7 | C94S MmUBC6 | C89S MmUBC7 |
|------------|-------------|-------------|-------------|-------------|
| 1          | 3.5         | 3.5         | 5.6         | 5.8         |
| 2          | 3.5         | 3.5         | 5.2         | 5.0         |
| 3          | 4.1         | 3.6         | 6.4         | 6.3         |
A

\[ \begin{array}{|c|c|c|}
\hline
& K>R TCR\alpha & + \\
\hline
C_{go}SMmUBC7 & - & + \\
\hline
IP: Anti-TCR\alpha & 46 & - \\
IB: Anti-TCR\alpha & 30 & - \\
\hline
K=R TCR\alpha & - & + \\
C_{go}SMmUBC7 & - & + \\
LCN & - & + \\
\hline
IP: Anti-TCR\alpha & 49 & - \\
IB: Anti-TCR\alpha & 36 & - \\
\hline
\end{array} \]

**Fig. 8.** Effect of inactive MmUBC7 on levels of lysine-less TCR-\alpha. A, HEK-293 cells were transfected with Lysine-less (K=R) TCR-\alpha with or without C_{go}SMmUBC7. Immunoprecipitation (IP) was carried out with anti-TCR-\alpha antibody followed by immunoblotting (IB) with the same antibody. Blots were developed using 125I-labeled protein A. B, HEK-293 cells were transfected as in A, and cells in lanes 3 and 5 were treated with lactacystin. Immunoprecipitation and blotting was done as in A. Faster migrating forms are indicated with an arrow. Lower panel shows immunoblot with anti-GFP antibody for control of transfection efficiency.

Consistent with this, TCR-\alpha treated with N-glycanase comigrates with the lower molecular weight forms of TCR-\alpha (Fig. 7B).

To determine whether the inhibitory effects on TCR-\alpha degradation observed with C_{go}SMmUBC7 are dependent on ubiquitination of TCR-\alpha on lysine residues, we evaluated a previously reported lysine-less form of TCR-\alpha (27). This form of TCR-\alpha is degraded from the ER in a proteasome-dependent manner despite a lack of potential sites for ubiquitination. As is evident, lysine-less TCR-\alpha also accumulated when inactive MmUBC7 is coexpressed (Fig. 8A). Treatment with lactacystin results in accumulation of lysine-less TCR-\alpha, including lower molecular weight forms. As with wild type TCR-\alpha, appearance of these forms is decreased when C_{go}SMmUBC7 is coexpressed (Fig. 8B), and, as would be predicted, these species were PnK-sensitive (data not shown).

\textit{C}_{go}SMmUBC7 Has Dominant Negative Effect on CD3-\delta Degradation—The CD3-\delta subunit of the TCR is also a substrate for ERAD when it fails to assemble with other TCR components and exit the ER (14). This protein differs from TCR-\alpha in having a substantial cytoplasmic domain and only a single charge in its transmembrane domain. To assess whether MmUBC7 is also involved in the degradation of this protein, pulse-chase analysis of CD3-\delta was carried out. When CD3-\delta was expressed alone in HEK-293 cells, it was rapidly degraded (Fig. 9A). However, as with TCR-\alpha, its loss was significantly inhibited when coexpressed with catalytically inactive MmUBC7. Inactive MmUBC6 did not significantly affect CD3-\delta degradation (not shown).

To further evaluate CD3-\delta a C-terminal HA-tagged form was generated. After determining by pulse-chase metabolic labeling that it was degraded with kinetics indistinguishable from wild type CD3-\delta (data not shown), it was used to assess the effects of catalytically inactive MmUBC7 on steady state levels of CD3-\delta by Western blotting. As expected, steady state levels of CD3-\delta increased either when catalytically inactive MmUBC7 was overexpressed or when proteasome function was inhibited (Fig. 9B).

Unlike TCR-\alpha, for which retrotranslocated forms are detectable when proteasome function is inhibited, our previous studies in T cells had not noted any evidence of retrotranslocation of endogenous CD3-\delta (14). Consistent with this, regardless of whether or not inactive MmUBC7 was coexpressed with CD3-\delta, forms that accumulated in the presence of proteasome inhibitor were limited in distribution to the membrane fraction. These results are in accord with the model of coupled extraction and proteasomal activity previously suggested for CD3-\delta and suggest that, as with TCR-\alpha, MmUBC7 is acting prior to retrograde movement through the ER membrane (Fig. 9C).

**DISCUSSION**

This study provides evidence that two mammalian E2s localize to the ER membrane. For MmUBC6 its C-terminal hydrophobic domain provides a basis for its membrane insertion. MmUBC7 lacks a membrane anchor that would allow for direct membrane insertion. In yeast, interaction with a C-terminal anchored protein, Cue1p, provides a molecular explanation for ER localization of UBC7p (11). Although mammalian Cue1p homologs have not been reported, it seems likely that an analogous protein may play a role in tethering of MmUBC7 to the ER membrane.

Previous studies in cells expressing a temperature-sensitive ubiquitin activating enzyme (E1) have shown that a functional Ub pathway is required for degradation of TCR-\alpha from the ER (27). Data presented herein provides the first evidence implicating specific E2, MmUBC7, in degradation from the ER in mammalian cells. Overexpression of catalytically inactive MmUBC7 results in decreased degradation of both the TCR-\alpha and the CD3-\delta subunits of the TCR. In contrast there is no evidence of a role for MmUBC6 in degradation of TCR-\alpha. Similarly, no effect of mutant MmUBC6 was observed on degradation of CD3-\delta.\(^2\) The negative data obtained with inactive MmUBC6 is consistent with deletion analyses in yeast where UBC7p is the predominant E2 in ERAD, whereas UBC6p has partial effect on degradation of certain proteins, including Sec61 (6) and CPY\(^\ast\) (7), and no effect on Vph1 (28).

For proteins such as CD3-\delta, which have cytoplasmically disposed lyses, it is easy to envisage models for ERAD that include ubiquitination of cytoplasmic lysines, recognition of ubiquitinated species by proteasomes, and dislocation and destruction from the ER facilitated by chaperone-like functions of proteasome. The lack of discernable retrotranslocation observed for CD3-\delta in the presence of lactacystin with or without coexpression of inactive MmUBC7 is consistent with such a model and extends previous observations from our laboratory made on endogenous CD3-\delta in T cells. Less obvious is how components of the Ub-conjugating system function in the retrotranslocation of lumenal proteins and of transmembrane proteins lacking cytoplasmic sites for ubiquitination, such as TCR-\alpha. The N terminus of this protein is in the ER lumen, and it has no lysines in its cytoplasmic tail. When evaluated by pulse-chase analyses in T cells, TCR-\alpha undergoes a discrete degree of proteasome-independent retrograde translocation that should allow exposure of potential sites of ubiquitination (14). However, similar evidence for partial retrograde movement is not obvious in our experiments in HEK-293 cells. Studies from other groups carried out in non-T cells have provided evidence that when expressed ectopically some level of complete retrograde translocation and accompanying deglycosylation of TCR-\alpha occurs in the absence of proteasome function (29, 30). Our observations corroborate these findings. How-

\(^2\) S. Tiwari and A. M. Weissman, unpublished observations.
FIG. 9. Effect of C89SMmUBC7 on half-life of CD3-δ. Pulse-chase analysis of CD3-δ alone or cotransfected with Myc-C89SMmUBC7. Lower panel shows sequential immunoprecipitation (IP) with anti-HA antibody for MmUBC7 expression. Graph shows the degradation curve for CD3-δ from this experiment with 100% representing CD3-δ at 0 h time point. Open circles, CD3-δ alone; closed circles, CD3-δ cotransfected with C89SMmUBC7. B, HEK-293 cells transfected with HA-CD3-δ-pCI alone or with Myc-C89SMmUBC7 were treated with lactacystin (LCN) for 12 h and immunoprecipitated with anti-HA antibody and blotted with anti-CD3-δ antibody. Lower panel shows anti-Myc and anti-GFP blots of the whole cell lysate to show MmUBC7 expression and loading control, respectively. C, Cells transfected as in B were treated with lactacystin (LCN) and lysed without detergent. After pelleting unbroken cells and nuclei, cytosolic (C) and membrane (M) fractions were prepared. Samples were immunoprecipitated with anti-HA antibody and blotted with anti-CD3-δ antibody. IB, immunoblotting.

Nevertheless, even after 16 h of proteasome inhibition, the amount of cytoplasmically disposed TCR-α represents only a small fraction of the total accumulated material, suggesting a continued requirement for proteasome function for efficient complete retrotranslocation and degradation from the ER, as has been suggested for an engineered model substrate (31) and for Pdr5 (8) in yeast. Similarly, a requirement for proteasome function in retrotranslocation has been reported for unassembled soluble Ig subunits (32) and CD4 (33, 34) in mammalian cells. Results with TCR-α demonstrate that the relative amounts of cytoplasmically disposed material is consistently decreased when inactive MmUBC7 is coexpressed, even when proteasome function is inhibited, suggesting that MmUBC7 may play a role in the process leading to retrotranslocation upstream of the involvement of proteasomes. For CD3-δ the absence of detectable cytoplasmic forms under any circumstances precludes statements as to whether MmUBC7 act upstream of the proteasome. But it is evident that, as with TCR-α, this E2 is affecting the fate of CD3-δ prior to its removal from the ER membrane. Results obtained with both of these transmembrane TCR components are consistent with findings in yeast for a nontransmembrane ER protein, CPY*, where deletion of UBC7 resulted in its accumulation in the ER lumen (35). At least for nonmembrane proteins, SCF complex components are known to function with the yeast E2 CDC34 and its mammalian homologs. Whether mammalian homologs of HRD1 exist remains to be determined. Another ubiquitin ligase component implicated in degradation from the ER is the F-box protein βTRCP. This protein forms part of an SCF E3 complex and is implicated in the degradation of CD4 from the ER with phosphorylated HIV-1 Vpu functioning as an adaptor (33, 36). At least for nonmembrane proteins, SCF complex components are known to function with the yeast E2 CDC34 and its mammalian homologs. Whether βTRCP-containing SCF takes advantage of ER membrane-bound MmUBC7 or perhaps MmUBC6 when targeting CD4 for degradation from the ER now awaits determination.

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