Evidence That Endoplasmic Reticulum (ER)-associated Degradation of Cystic Fibrosis Transmembrane Conductance Regulator Is Linked to Retrograde Translocation from the ER Membrane*

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The ubiquitin-proteasome pathway has been implicated in the degradation of newly synthesized, misfolded and unassembled proteins in the endoplasmic reticulum (ER). Using a cell-free reticulocyte lysate system we have examined the relationship between biosynthesis and ER-associated degradation of the cystic fibrosis transmembrane conductance regulator (CFTR), a polytopic protein with 12 predicted transmembrane segments. Our results provide direct evidence that full-length, glycosylated and membrane-integrated CFTR is a substrate for degradation and that degradation involves polyubiquitination and cytosolic proteolytic activity. CFTR ubiquitination was both temperature- and ATP-dependent. Degradation was significantly inhibited by EDTA, apyrase, and the proteasome inhibitors hemin and MG132. Degradation was inhibited to a lesser extent by clasto-lactacystin β-lactone, ALLN, and N-tosyl-l-phenylalanine chloromethyl ketone and was relatively unaffected by lactacystin and N-tosyl lysyl chloromethyl ketone. In the presence of hemin, polyubiquitinated CFTR remained tightly associated with ER microsomes. However, membrane-bound ubiquitinated CFTR could be subsequently degraded into trichloroacetic acid-soluble fragments upon incubation in hemin-free, ATP-containing lysate. Thus ER-associated degradation of CFTR occurs via a membrane-bound, rather than cytosolic, intermediate and likely involves recruitment of degradation machinery to the ER membrane. Our data suggest a model in which the degradation of polytopic proteins such as CFTR is coupled to retrograde translocation and removal of the polypeptide from the lipid bilayer.

The rough endoplasmic reticulum (ER)1 facilitates translocation, membrane integration, folding, and oligomeric assembly of most proteins found in the secretory pathway of eukaryotic cells (1-4). The ER also functions to recognize misfolded or unassembled proteins and prevent their exit to the Golgi complex (5-7) through a process termed ER-associated degradation (ERAD) (8). Substrates for ERAD, like other secretory and transmembrane proteins, are initially targeted to and translocated across the ER membrane via the Sec61 translocon complex (1, 2). However, aberrantly folded proteins are selectively translocated back into the cytosol where they undergo ubiquitination and degradation by cytosolic proteases (9-11). Certain ERAD substrates such as major histocompatibility complex class I heavy chains (10, 12, 13) and unglycosylated prepro-α factor undergo complete retrograde translocation into a soluble, cytosolic form prior to degradation (8). In contrast, mutant forms of carboxypeptidase Y and unassembled T cell receptor α subunits translocate back to the cytosol but remain associated with cytoplasmic face of the ER membrane (14, 15). In both cases the generation of cytosolic intermediates is thought to occur via retrograde transport through the Sec61 translocon complex (9-11).

The ubiquitin-proteasome pathway is also involved in the ER-associated degradation of polytopic proteins. Yeast mutants with defective ubiquitin-conjugating enzymes or proteasome subunits show decreased degradation of mutant forms of Sec61p (16, 17), a resident ER protein with 10 predicted transmembrane segments (18). ERAD may also play a role in ensuring proper protein levels in the ER as suggested by mevalonate-mediated regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (19-21). For the cystic fibrosis transmembrane conductance regulator (CFTR), a plasma membrane protein, approximately 80% of wild type protein and 100% of common mutant forms are degraded prior to reaching the Golgi complex (22, 23). Recent studies have demonstrated that ER-associated degradation of CFTR also requires an intact ubiquitin conjugation pathway and likely involves the 26 S proteasome (24, 25). While the underlying reasons for CFTR degradation remain unknown, it has been proposed that the majority of wild type CFTR, like mutant CFTR, is recognized by ER quality control machinery, because it fails to fold properly (26-29).

Degradation of polytopic proteins by cytosolic proteases poses a particular problem in that multiple transmembrane (TM) segments must be removed from the membrane. It is unclear whether polytopic proteins, like secretory and bitopic transmembrane proteins, proceed through cytosolic intermediates prior to degradation. One possibility is that the proteasome might degrade only cytosolic peptide loops, leaving luminal and/or transmembrane segments to be degraded by other proteases (7, 30). Alternatively, degradation might be coupled to the process of retrograde translocation. To distinguish among these possibilities, it will be necessary to define the temporal relationship between recognition, retrograde translocation, and degradation events involved in polytopic protein ER
degradation. In this regard, a cell-free system that reconstitutes ERAD is attractive, because it is readily amenable to physical and pharmacological manipulation. Using such a system, Sato et al. (31) recently demonstrated that CFTR could be ubiquitinated cotranslationally, indicating that initial recognition events in ER degradation might occur very early in the lifetime of a polytopic protein. In the current study we use a similar cell free system to demonstrate that CFTR ubiquitination can also occur after protein synthesis, N-linked glycosylation, and membrane integration have been completed. We have also been able to separate the process of ubiquitination from that of degradation and now show that ubiquitinated CFTR remains tightly associated with the ER membrane until it is degraded into trichloroacetic acid-soluble peptide fragments. CFTR degradation is therefore physically localized to the ER. Our results are consistent with a model in which cytosolic degradation machinery is recruited to the ER membrane by polyubiquitinated substrates and suggest that degradation occurs coincident with poly peptide removal from the lipid bilayer.

**EXPERIMENTAL PROCEDURES**

**Materials—** ALN, TPCK, TLCK, hemin, bovine ubiquitin, anti-bovine ubiquitin antibody, and 14C-methylated lysylase were purchased from Sigma. The protease inhibitor mixture, (4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, E-64, EDTA, and leupeptin), MG132, lactacystin, and clasto-lactacystin β-lactone were purchased from Calbiochem. Nucleotide triphosphates, creatine phosphate, creatine kinase, and diithiothreitol (DTT) were purchased from Boehringer Mannheim. Anti-CFTR antisera was raised against synthetic peptides corresponding to residues 45–65 (N terminus), 680–700 (R domain), and 1458–1518 (C terminus). The presence of full-length CFTR protein in a cell-free RRL system, and membrane pellets were dissolved directly in SDS loading buffer. Supernatants were precipitated in 20% trichloroacetic acid, pelleted at 16,000 × g for 15 min, washed with acetone, and dissolved in SDS loading buffer.

**Immunoprecipitation—** RRL aliquots were diluted directly into 1 ml of ice-cold Buffer A (0.1 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM Tris (pH 8.0)) and preincubated with antisera for 10–30 min prior to addition of 5 μl of protein A Affi-Gel (Bio-Rad). CFTR antisera and nonimmune antisera were used at 1:1000 dilution. Ubiquitin antibody was used at 1:200 dilution (similar amounts of Ig were present in all reactions). Samples were mixed at 4°C for 6–10 h, washed three times with Buffer A, twice with 0.1 mM NaCl, 0.1 mM Tris (pH 8.0), and taken up in SDS loading buffer.

**Degradation Assay—** The translation mixture was layered onto a 0.5 mM sucrose buffer (0.5 mM sucrose, 50 mM HEPES (pH 7.5), 0.1 mM KCl, 5 mM MgCl2, and 1 mM DTT) and centrifuged at 180,000 × g for 10 min. Membranes were resuspended in ice-cold 0.1 mM sucrose buffer (0.1 mM sucrose, 50 mM HEPES (pH 7.5), 0.1 mM KCl, 5 mM MgCl2, and 1 mM DTT) at one-half the original translation volume. The degradation reaction, assembled on ice, included 20% resuspended membranes, 72% reticulocyte lysate, and a final concentration of 10 mM Tris (pH 7.5), 1 mM ATP, 5 mM MgCl2, 12 mM creatine phosphate, 3 mM DTT, and 80 μg/ml of creatine kinase. Where indicated, RRL was replaced with water, and proteasome inhibitors were added. For ATP-depleted RRL, ATP and creatine phosphate were omitted. The degradation mixture was incubated at 37°C, and aliquots were added directly to loading buffer for SDS-PAGE. EnHance (NEN Life Science Products) fluorography and autoradiography. Autoradiograms were scanned on an AGFA studioscan II transmission scanner using Adobe Photoshop software. Quantitation of autoradiograms was performed using an Amersharm Pharmacia Biotech DTS scanning densitometer precalibrated with a Kodak photographic step tablet (Eastman Kodak Co.). Trichloroacetic acid-soluble counts were determined by scintillation analysis using a Beckman LS6500 scintillation counter.

**vesicle Pelleting and Floation—** Microsomal membranes from translation mixture were pelleted, resuspended, and incubated in ATP- and creatine phosphate buffered 0.1 mM NaCl, 0.1 mM Tris (pH 7.5), 1 mM MgCl2, and 1 mM DTT at 37°C. Aliquots of the RRL/hemin mixture were incubated at 37°C for 90 min (33). Reticulocyte lysate, and a final concentration of 10 mM Tris (pH 7.5), 1 mM ATP, 5 mM MgCl2, 12 mM creatine phosphate, 3 mM DTT, and 80 μg/ml of creatine kinase. Where indicated, RRL was replaced with water, and proteasome inhibitors were added. For ATP-depleted RRL, ATP and creatine phosphate were omitted. The degradation mixture was incubated at 37°C, and aliquots were added directly to loading buffer for SDS-PAGE. EnHance (NEN Life Science Products) fluorography and autoradiography. Autoradiograms were scanned on an AGFA studioscan II transmission scanner using Adobe Photoshop software. Quantitation of autoradiograms was performed using an Amersharm Pharmacia Biotech DTS scanning densitometer precalibrated with a Kodak photographic step tablet (Eastman Kodak Co.). Trichloroacetic acid-soluble counts were determined by scintillation analysis using a Beckman LS6500 scintillation counter.

**In Vitro CFTR Biogenesis—** To examine the steps of CFTR degradation in native ER membranes, we first characterized expression of full-length CFTR protein in a cell-free RRL system. Translation of plasmid pSPCFTR (35) in the absence of transcription mixture in 20% trichloroacetic acid, incubating on ice for 30 min, pelleting at 16,000 × g for 15 min, and counting supernatants in 10 volumes of Ready Safe scintillation fluid (Beckman, Fullerton, CA) in a Beckman LS6500 scintillation counter.

**RESULTS**

In Vitro CFTR Biogenesis—To examine the steps of CFTR degradation in native ER membranes, we first characterized expression of full-length CFTR protein in a cell-free RRL system. Translation of plasmid pSPCFTR (35) in the absence of ER-derived microsomal membranes generated a polypeptide migrating at ~160 KDa (Fig. 1A). This translation product was shifted 6 KDa in size. Three rough microsomes were added at the start of translation, consistent with the addition of two N-linked core carbohydrates at the predicted consensus sites in the fourth extracytoplasmic loop. The presence of full-length CFTR was confirmed by immunoprecipitation with peptide-specific antisera raised against N terminus, R domain, and C terminus of CFTR. The presence of full-length CFTR was confirmed by immunoprecipitation with peptide specific antisera raised against N terminus, R domain, and C terminus of CFTR. The presence of full-length CFTR was confirmed by immunoprecipitation with peptide specific antisera raised against N terminus, R domain, and C terminus of CFTR. The presence of full-length CFTR was confirmed by immunoprecipitation with peptide specific antisera raised against N terminus, R domain, and C terminus of CFTR. The presence of full-length CFTR was confirmed by immunoprecipitation with peptide specific antisera raised against N terminus, R domain, and C terminus of CFTR.
terminus epitopes. Carbonate extraction of translation products demonstrated that in vitro synthesized CFTR was fully integrated into the ER membrane. Thus the RRL system is capable of efficiently reconstituting the early events of CFTR biosynthesis and processing, in agreement with results reported by Sato et al. (31). To test the stability of newly synthesized CFTR in the ER membrane, cyclohexamide was added after 2 h of translation, and samples were incubated for an additional 4 h (Fig. 1B). At 24 °C, CFTR was remarkably stable, whereas at 37 °C CFTR was rapidly converted into a high molecular weight (HMW) complex with an estimated size of >450 kDa ($T_{1/2}$, 30 min). For convenience, this large complex will subsequently be referred to as the HMW complex, and the ~170 kDa band will be referred to as full-length CFTR.

**ATP-dependent Conversion of CFTR into a HMW Complex**—To determine the requirements for conversion of CFTR into the HMW complex, CFTR was translated in RRL, and microsomal membranes were collected by pelleting through a sucrose cushion (Fig. 2A). Microsomes were then resuspended and incubated at 37 °C in sucrose buffer containing ATP (lanes 1–5), RRL supplemented with an ATP regeneration system (lanes 6–10), ATP-depleted RRL (lanes 11–15), or ATP-depleted RRL supplemented with additional ATP (lanes 16–20). At specified time points, samples were analyzed directly by SDSPAGE. In the presence of buffer alone, or in ATP-depleted RRL, >70% of full-length CFTR remained intact after 4 h of incubation (Fig. 2B). However, when microsomal membranes were incubated in fresh RRL containing ATP, or in ATP-depleted RRL supplemented with additional ATP, full-length CFTR was rapidly converted into the HMW complex ($T_{1/2} < 30$ min) that subsequently disappeared over the next 4 h. These results are consistent with a two-step process in which full-length CFTR is initially converted into a HMW complex via an ATP-dependent mechanism, and the HMW complex is then a substrate for degradation.

**The CFTR HMW Complex Is Polyubiquitinated**—We noted that degradation of the CFTR HMW complex in Fig. 2 contrasted with results in Fig. 1B where the complex accumulated over time. One difference between these two experiments is
containing newly synthesized CFTR were resuspended in 0.1 M sucrose buffer and incubated at 37 °C in RRL containing 40 μM hemin and 0.4 mg/ml of bovine ubiquitin (A) or methylated ubiquitin (B). At times indicated, samples were immunoprecipitated with nonimmune sera (NIS; A, lanes 6–10), anti-ubiquitin antisera (A, lanes 11–15), or analyzed directly (A, lanes 1–5 and B, all lanes).

that CFTR translation is performed in the presence of hemin, a required co-factor for protein expression in RRL (38), and aurin tricarboxylic acid, an inhibitor of protein synthesis initiation. Both of these compounds inhibit ATP-dependent degradation of ubiquitinated substrates in RRL. These results, together with the requirement for ATP and cytosol, suggested that the HMW complex might represent polyubiquitinated CFTR. Microsomal membranes containing newly synthesized CFTR were therefore incubated in fresh RRL supplemented with bovine ubiquitin and 40 μM hemin. Aliquots were then immunoprecipitated with anti-bovine ubiquitin antisera and analyzed by SDS-PAGE at specified time points. As shown in Fig. 3A, the HMW complex, but not full-length CFTR, was reactive against anti-ubiquitin antisera (lanes 11–15). No reactivity was observed when immunoprecipitation was performed with nonimmune sera (NIS, lanes 6–10) or when bovine ubiquitin was omitted from the lysate (data not shown). To further confirm CFTR ubiquitination, microsomes containing newly synthesized CFTR were incubated in RRL in the presence of methylated ubiquitin, which blocks the sequential addition of ubiquitin molecules by preventing isopeptide bond formation at ubiquitin residue Lys-48. Under these conditions we observed a dose-dependent inhibition in the rate of HMW complex formation (Fig. 3B). Taken together, these data demonstrate that the sequential, covalent addition of multiple ubiquitin molecules contributes to the formation of the CFTR HMW complex.

Inhibition of CFTR HMW Complex Degradation by Proteasome Inhibitors—We next examined the ability of proteasome inhibitors to influence the disappearance of the CFTR HMW complex. Inhibitors of serine, cysteine, and metalloproteases had essentially no effect on the rate of disappearance of either full-length CFTR or the HMW complex (Fig. 4A). In contrast, hemin and MG132, both known inhibitors of the proteasome, resulted in significant delay in the disappearance of the HMW complex particularly at early time points (Fig. 4B). Quantitation of autoradiograms revealed that hemin effectively blocked 60% of HMW complex degradation. In contrast, MG132, ALLN, and TPCK slowed but did not prevent HMW complex disappearance. Surprisingly, little inhibition was observed for lactacystin or TLCK. These results suggested either that certain proteasome inhibitors might be relatively inactive in the RRL system, or alternatively, that degradation of ubiquitinated CFTR might involve additional protease activities. To distinguish between these possibilities, we therefore examined the effect of proteasome inhibitors on the degradation of methylated lysozyme, a known substrate of the 26 S proteasome. For these and subsequent experiments the rate and extent of protein degradation was quantitated by measuring the conversion of CFTR and/or lysozyme into trichloroacetic acid-soluble peptide fragments.

As shown in Fig. 5, 26% of [14C]lysozyme and 65% of newly synthesized CFTR were degraded into trichloroacetic acid-soluble peptide fragments within 3 h in RRL. Lysozyme degradation was inhibited by approximately 90% in the presence of 40 μM hemin or 10 mM EDTA consistent with previous studies (39). Hemin also resulted in formation of a HMW complex containing [14C]lysozyme (data not shown). In contrast, the specific proteasome inhibitor lactacystin, as well as its active metabolite clasto-lactacystin β-lactone, had little effect on lysozyme degradation (<10% inhibition). Under these same conditions CFTR degradation was inhibited by approximately 80% in the presence of EDTA or the ATP hydrolyzing enzyme, apyrase. Hemin inhibited CFTR degradation by 56%, while lactacystin and clasto-lactacystin β-lactone inhibited CFTR degradation by only 20 and 35%, respectively. In these latter experiments, lactacystin-based inhibitors were preincubated with RRL for 30–60 min prior to addition of CFTR-containing microsomal membranes. These experiments demonstrate that specific lactacystin-based proteasome inhibitors are relatively inactive at blocking degradation of these ubiquitinated substrates in RRL. It should also be noted that a significant amount of CFTR degradation in RRL (35–40%) appears to proceed through a hemin insensitive pathway distinct from that utilized by lysozyme.

Ubiquitinated CFTR Remains Tightly Associated with the ER Membrane—Sixty-five percent of in vitro synthesized CFTR protein was degraded to trichloroacetic acid-soluble fragments within 3 h of incubation in RRL (Fig. 5). Degradation could not be restricted solely to large cytosolic peptide loops (i.e. N terminus, nucleotide binding domains, and the R-domain), as these loops contain only 47% of total methionine residues (40). Rather, our data indicate that proteolytic machinery also gains access to small cytosolic loops and possibly luminal and/or transmembrane regions of the protein. Thus prior to or coincident with degradation, some or all of CFTR is likely to be extracted from the membrane. We therefore tested whether the ubiquitinated HMW complex, which has already been targeted for degradation by ubiquitin-conjugating enzymes, might undergo retrograde translocation to a cytosolic intermediate. Ubiquitinated CFTR complexes were generated by incubation in RRL (in the presence of hemin), and vesicles were collected by pelleting through a sucrose cushion. As shown in Fig. 6A, both CFTR and the CFTR HMW complex were nearly quantitatively recovered in the microsome pellet in the absence of detergent. In addition, preincubation of CFTR-containing microsomes in high salt (up to 1.1 M KCl) failed to extract full-length CFTR or the HMW complex into the supernatant fraction (Fig. 6B). Thus ubiquitinated CFTR does not behave as a cytosolic or peripheral membrane protein.

Results from Fig. 6 do not rule out the possibility that HMW ubiquitinated CFTR might also be pelleted as a large, detergent-sensitive protein complex. We therefore tested whether the HMW complex was physically associated with the ER by membrane flotation. Aliquots of RRL containing newly syn-
thesized CFTR and/or polyubiquitinated CFTR were diluted into 2 M sucrose, layered beneath a discontinuous sucrose gradient, and centrifuged for 2 h as described under “Experimental Procedures” (Fig. 7A). For half of the samples, ribosomes were first stripped from membranes by incubation in KCl/EDTA (Fig. 7B). The secretory protein bovine prolactin (41) was used as a standard to determine the location of microsomal membranes in the gradient. Both full-length CFTR and the polyubiquitinated complex exhibited the same floatation pattern as the secretory control. Thus in the absence of degradation, CFTR remained membrane-associated long after ubiquitination had occurred. Following ribosome stripping with EDTA/KCl microsomes more uniformly partitioned into the lighter fractions. However ribosome removal had no detectable effect on dislocation of ubiquitinated CFTR from the ER membrane.

Degradation of Membrane-bound, Ubiquitinated CFTR—To test whether membrane-bound CFTR remained a substrate for degradation and not simply a protease resistant aggregate, CFTR-containing microsomes were incubated in RRL in the presence of hemin to allow accumulation of the ubiquitinated intermediate. Vesicles were isolated at sequential time points, reincubated in the presence or absence of fresh RRL, and degradation of CFTR was monitored by release of trichloroacetic acid-soluble counts. As shown in Fig. 8A, 70% of total CFTR was degraded into trichloroacetic acid soluble counts following 3 h incubation in ATP-containing RRL. When vesicles were collected after 20 min of preincubation in hemin-containing RRL and resuspended in fresh RRL lacking hemin, 56% of the total CFTR was subsequently degraded. This degradation remained sensitive to both hemin and EDTA. Incubation of CFTR in hemin-containing RRL for longer periods of time (i.e. 1–2 h) resulted in the near complete conversion (~90%) of full-length protein into the HMW complex as in prior experiments (data not shown). Even after 2 h of preincubation, 56% of total CFTR protein was still able to be degraded upon removal of hemin. In each of these experiments, the degradation of polyubiquitinated CFTR was thus 80% as efficient as the degradation of newly synthesized protein. Furthermore, with longer preincubation times the reversible component of CFTR degradation became progressively more sensitive to inhibition such that after 2 h of preincubation, hemin and EDTA each blocked degradation of polyubiquitinated CFTR by approximately 90%. When microsomes containing pre-ubiquitinated CFTR were similarly resuspended and incubated in the presence of buffer...
following formula: % counts released

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\text{Percent Soluble Counts Released} = \frac{C_n - C_0}{T - C_0}
\]

where \(C_n\) = soluble counts at each time point, \(C_0\) = soluble counts at \(T = 0\), and \(T\) = total counts per aliquot. Results represent the average (±S.E.) of two to six separate experiments.

alone (Fig. 8B), no degradation of the HMW polyubiquitinated CFTR was observed.

Together these results demonstrate that: (i) the inhibitory effects of hemin on CFTR degradation are reversible, (ii) most, but not all, of the membrane-bound ubiquitinated CFTR remains a substrate for degradation, (iii) CFTR degradation, like ubiquitination, requires the presence of both ATP and cytosolic factors, and (iv) degradation is physically localized to the ER membrane and likely occurs coincident with removal of at least a portion of CFTR from the lipid bilayer.

**DISCUSSION**

The ER is a primary site of cellular quality control involved in the recognition and disposal of misfolded and unassembled proteins (5). While it was initially proposed that ER proteases were responsible for degrading ER substrates, recent evidence indicates that ERAD is mediated largely by cytosolic proteases including the 26 S proteasome (6, 7). This topologic disparity between substrate and degradation machinery has resulted in a reevaluation of protein movement into and out of the ER compartment (6). It now appears that translocation across the ER membrane is a bidirectional process that is regulated, in part, by the folded state of a given protein substrate. Whereas, nascent secretory and transmembrane proteins are targeted to, translocated across, and/or integrated into the ER membrane by ER translocation machinery (2, 42), proteins that fail to fold properly may also be recognized by ER quality control machinery and transported out of the ER and back to the cytosol (6, 9, 10, 15). These observations have raised important questions regarding the process of retrograde transport across the ER membrane. At what stage of biogenesis are protein substrates identified? How do recognition events target proteins back to the translocon and/or facilitate movement into the cytosol? And how is this retrograde transport coupled to the process of degradation? In the current study, we address these issues by developing an in vitro system that reconstitutes de novo synthesis and ER-associated degradation of the polytopic protein CFTR. Full-length, glycosylated and membrane-integrated CFTR was a substrate for ATP-dependent ubiquitination. In addition, polyubiquitinated CFTR remained tightly associated with the ER membrane until it was degraded into trichloroacetic acid-soluble peptide fragments. Thus ubiquitination precedes retrograde translocation, and degradation of polytopic proteins such as CFTR is physically localized to the ER membrane rather than the cytosol as proposed for some secretory and transmembrane substrates (8, 10, 13).

Our results are consistent with a model in which CFTR degradation is tightly coupled to removal of the polypeptide from the lipid bilayer. At least 65% of [35S]methionine-labeled CFTR was degraded into trichloroacetic acid-soluble fragments, consistent with proteasomal degradation in which the resulting polypeptides are 6–9 residues in length (43). Because a small fraction of full-length CFTR (approximately 10–15%) remained intact in nearly all experiments, the extent of degradation for individual CFTR polypeptides actually approached 80%. Thus CFTR degradation could not be limited to large cytosolic regions containing the N terminus, nucleotide binding domains, and R domain, which together comprise 61% of the total mass but only 47% of methionine residues (40). If all cytosolic loops within 8–15 residues from the membrane were cleaved, then degradation of all full-length CFTR polypeptide chains would liberate only 60% total CFTR methionine into the trichloroacetic acid-soluble fraction (40). It therefore seems likely that proteolytic machinery such as the proteasome might participate in removal of CFTR from the lipid bilayer to gain access to transmembrane segments and/or luminal peptide regions. This possibility is supported by the observation that: (i) proteasome-like particles are physically localized to the cytosolic surface of the ER membrane (44) and (ii) our results that CFTR degradation occurs via a membrane-bound, rather than cytosolic, intermediate. If retrograde translocation of CFTR occurred through the Sec61 translocon, then extraction from the lipid bilayer could potentially involve the reverse process of membrane integration, namely lateral movement of TM segments from the membrane back into the translocation channel and into the cytosol (45, 46).

While this study provides additional support that the ubiquitin-proteasome pathway is involved in ER-associated degradation of CFTR, it is unclear why different proteasome inhibitors exhibited different effects on CFTR degradation. Lactacystin and clasto-lactacystin β-lactone, which covalently inactivate the active site threonine residue on the β5 subunit of the 20 S proteasome (subunit X of the mammalian proteasome)
had relatively minor effects on CFTR degradation as determined either by disappearance of the CFTR HMW complex (Fig. 4) or generation of trichloroacetic acid-soluble fragments (Fig. 5). It is interesting that clasto-lactacystin β-lactone, the active form of lactacystin, is inactivated by glutathione in cells (49, 50). Our observation that neither lactacystin nor its β-lactone form inhibited the degradation of lysozyme, a known proteasome substrate, suggests that RRL is capable of rendering these agents inactive, even when preincubated in the absence of exogenous reducing agents. We do not know why the results reported here differ from those of Qu et al. (51) that demonstrated 20 μM lactacystin inhibited the degradation of both α1-antitrypsin and lysozyme in a similar RRL system. However, our results were consistent for two different preparations of lactacystin and at multiple inhibitor concentrations up to 100 μM. It is possible that these differ-

**Fig. 6.** HMW complex pelleting. CFTR-containing microsomes were incubated in RRL in the presence of hemin. A, at times specified, aliquots were analyzed directly (lanes 1–4), pelleted through 0.5 M sucrose buffer (lanes 5–9), or incubated on ice in 1% Triton X-100 for 30 min prior to pelleting (lanes 10–14). Pellets were dissolved directly into SDS loading buffer. B, aliquots of RRL degradation mixture were taken at specified times and analyzed directly in SDS loading buffer (lanes 1–3) or incubated for 30 min on ice following addition of KCl to the final concentrations indicated (lanes 4–21). Samples were then pelleted through 0.5 M sucrose buffer, and equal fractions of pellet and supernatant were analyzed by SDS-PAGE.

**Fig. 7.** HMW complex floatation. Microsomal membranes containing newly synthesized CFTR or bovine prolactin were incubated at 37 °C in RRL degradation mixture for 0, 20, or 120 min in the presence of hemin. A, samples were mixed to a final concentration of 2 M sucrose, layered beneath a discontinuous sucrose gradient, and centrifuged at 350,000 × g for 2 h as described under “Experimental Procedures.” B, EDTA (10 mM) and KCl (0.6 M) were added prior to centrifugation. Aliquots were taken from top to bottom of the gradient (lanes 1–10, respectively). The HMW complex, CFTR, and prolactin control are indicated.
ences reflect varying amounts of proteasome in different RRL preparations.

The most potent inhibitors of CFTR degradation were EDTA, apyrase, and hemin. EDTA and hemin have been shown previously to inhibit the degradation of other proteasome substrates in RRL by stabilizing HMW ubiquitin conjugates (39, 52, 53). Hemin, a heme precursor, exerts pleiotropic effects in RRL. Its stimulatory effect on protein translation (38) is proposed to be mediated by inactivation of the translation factor eif-2, possibly by inhibiting an endogenous RRL kinase (54, 55). In addition, hemin directly inhibits ATPase activities of the purified 19 S proteasome regulatory complex (PA700) as well as the 26 S proteasome itself (56). Because the catalytic core of the 20 S proteasome cannot accommodate folded or ubiquiti-
nated polypeptides, it has been proposed that ATPases contained within the 19 S regulatory subunit are required for substrate unfolding as well as deubiquitination (43, 48, 56–59). It is therefore likely that hemin inhibits proteasome-mediated CFTR degradation by blocking ATPase activity and thereby preventing CFTR unfolding and/or deubiquitination. If CFTR unfolding were required for removal from the bilayer, a mechanism that would explain the accumulation of membrane-bound, polyubiquitinated CFTR observed in the current study.

Our results do not rule out the possibility that additional peptidase activities either in the ER lumen or within the membrane also participate in CFTR degradation (43, 48, 56–59). It has been suggested previously (25, 60) that the membrane-associated polyubiquitinated substrate might consist of polyubiquitinated CFTR cleavage products that are then degraded by the ubiquitin-proteasome pathway.

Cell-free systems have significantly enhanced our understanding of protein folding (64) and translocation (2). However, we do not yet know whether the folded state of newly synthesized CFTR in the RRL system is the same as that found in the ER of cells or whether the mechanism of CFTR recognition by ER quality control machinery in vitro is the same as in vivo. Processing in the RRL system is limited to early events that occur at the ER membrane, and it is possible that CFTR might require specialized cellular components that are limiting in RRL and/or canine pancreas microsomes. Recently, RRL and yeast-derived cell-free systems have been used to examine the degradation of other secretory and transmembrane proteins (8, 51), including CFTR (31). Our observation that 85–90% of newly synthesized wild type CFTR is degraded in the RRL system is consistent with studies that demonstrate most wild type CFTR is also degraded in the ER membrane of intact cells (22, 23). In addition, preliminary experiments in our laboratory have demonstrated that other polytopic proteins expressed in the RRL system, such as human P-glycoprotein and aquaporin water channels, are much more stable than CFTR (52, 53). These studies indicate that rapid CFTR degradation in vitro is not simply a cell-free artifact, but rather reflects specific structural features recognized by ER quality control machinery. In vitro systems such as RRL will thus likely provide a versatile tool for investigating the events and cellular machinery involved in

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ER-associated degradation. In the case of CFTR, they may also provide screening assays to identify compounds that improve folding and trafficking efficiency.

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